

**The conservation biology of *Pittosporum obcordatum*: conservation genetics and habitat specificity.**

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## Abstract

*Pittosporum obcordatum* (Pittosporaceae) is a threatened lowland shrub, primarily found in alluvial sites with widely separated populations throughout the North and South Islands of New Zealand. Threats to *P. obcordatum* include small population size, habitat loss, competition with exotic plants and grazing from introduced mammals. The primary goal of this thesis was to better understand genetic and ecological factors that could assist with the conservation of *P. obcordatum*. I used Inter-Simple Sequence Repeat (ISSR) markers to study the genetic diversity of 128 individuals from 10 populations of *P. obcordatum* from Kaitaia to Fiordland. Populations of *P. obcordatum* had low to moderate genetic diversity, and smaller populations contained less genetic diversity than larger populations. A high degree of geographic genetic structure was found, suggesting little or no recent gene flow between populations. Small population sizes and geographic isolation of the populations likely have an effect on this. Based on this data, recommendations are made for site-specific restoration planting designed to maximise population size and genetic diversity, accompanied by conservation strategies targeting threats from land use. Further, a newly discovered population of *P. obcordatum* on Banks Peninsula has been found growing on a hillside, and this thesis investigated why the species was growing in an unexpected habitat. When pot-grown seedlings from Banks Peninsula and a Fiordland alluvial flat population were subjected to drought, mortality was lower for Banks Peninsula seedlings than for Fiordland but not significantly so. When radishes (*Raphanus sativus*) were used as a phytometer, Banks Peninsula soil produced significantly less biomass than soil from three alluvial flats. Taken together, these findings indicate that slope and soil fertility have less of an impact on the success of *P. obcordatum* than would previously have been assumed based on its alluvial sites, indicating that further populations may yet be found on other hillsides or in less fertile areas. Search and further genetic study of such populations are therefore recommended in order to elaborate on the habitat and genetic profile advanced by this research, allowing for a well-rounded approach to conservation planning.

## Chapter 1: General Introduction

### Plant conservation in New Zealand

New Zealand is considered a biodiversity 'hotspot' (Brooks et al. 2002), due to its large number of endemic species and the comparatively small range size of these species. New Zealand has 1,856 endemic plant species, mainly centred in the northern North Island, and the northern and southern ends of the South Island (Wardle 1963; de Lange et al. 2010). Of the country's endemic plant species, 180 (7.6%) are considered 'Threatened' with a further 651 (27.6%) species considered 'At Risk' (Myers et al. 2000; Rogers & Walker 2002; de Lange et al. 2008; de Lange et al. 2013). In addition to high species endemism, the New Zealand flora has many rare species (22%), and is depauperate in annuals and deciduous trees (Myers et al. 2000; Rogers & Walker 2002). In contrast to other countries, which often have high numbers of rare species at medium to high elevations (e.g. France; Lavergne et al. 2004), New Zealand has a higher proportion of rare species in lowland and coastal areas. A large proportion of these are trees and shrubs (Rogers & Walker 2002; McGlone et al. 2010). Of the rare lowland taxa, 125 species are 'Threatened' and 63 species are 'Naturally Uncommon' (Rogers & Walker 2002).

In New Zealand, the primary threats to plant biodiversity are habitat loss, fragmentation and invasive species (Norton & Miller 2000). Following human settlement, native forest diminished from covering 82% of New Zealand to only 23% (Ewers et al. 2006). Only a relatively small proportion of habitats of threatened plants are under protection, with about 30% of New Zealand's land mass designated as either conservation estates or reserves (Norton & Miller 2000). However, these protected areas are biased towards upland regions that are not of productive value and are therefore of low economic value. As a result, protection is in place for 49% of upland areas > 500 m, as opposed to 18% of land below this elevation (Norton & Miller 2000). The ratio of highland to lowland conservation areas does not reflect the conservation needs of the New Zealand flora, as 30% of threatened indigenous plants are found only in lowland areas, and 20% of these occur only on private land (Norton & Miller 2000; de Lange et al. 2009).

Due to a lack of protection, New Zealand's lowlands are heavily affected by agriculture and invasive species (Green & Clarkson 2005). Most of the areas in which lowland forest once occurred are now cleared - largely for commercial purposes, typically agriculture (MacLeod & Moller 2006). Consequently, the remnants of New Zealand's indigenous lowland forest tend to be small isolated fragments, which are then further affected by human disturbance (Norton & Miller 2000; Dodd et al. 2011).

Introduced species of plants and animals also have an impact on plant biodiversity. New Zealand's plant species evolved without mammalian herbivores and have been significantly affected by a decrease in available habitat and an increased extinction risk as a result of introduced herbivore grazing (Nugent et al. 2001). Furthermore, many naturalised plant species out-compete endemic plant species, especially in habitats with high disturbance (Jesson et al. 2000; Sullivan et al. 2005). For example, Marram grass (*Ammophila arenaria*) displaces Pingao (*Ficinia spiralis*) on sand dunes throughout New Zealand (Hilton et al. 2005).

## Rare species

Determining if a species is naturally rare or rare as a result of human activities is important when assessing if a given species should be considered 'At Risk' and in need of conservation management. Rabinowitz (1981) developed a framework for rarity that categorises definitions by abundance, habitat range and geographic distribution. This framework encompasses eight modes, where a taxon is considered 'common' – that is, not rare - and the other modes denote varying degrees of rarity. Abundance is a simple measure of the size of the population/s of a species. Habitat range indicates the specificity of habitat the plant requires for growth: the wider the range, the less specific habitat the plant requires. Geographic distribution is a function of the plant's spread across the environment.

All eight modes of Rabinowitz's (1981) rarity classification have some combination of these three factors, with the most common category being used for taxa that are very abundant, occur in a wide range of habitats and have a wide geographic distribution. At the other end of the continuum are species that are scarce and restricted to a specific habitat type and one geographic location. The most well-documented cause of plant rarity is confinement to restricted habitats, where plants are habitat specialists (Kruckeberg & Rabinowitz 1985; Harrison 1999; Boulangeat et al. 2012). In this framework, the rarest taxon would have a narrow habitat spread, small population size and be constrained in its geographic distribution to a single area, as in the case of New Zealand's *Pittosporum ellipticum* subsp. *serpentinum*, which is confined to an ultramafic habitat on the Surville Cliffs, Northland (de Lange 1998).

Robbirt et al. (2006) critiqued Rabinowitz's framework of rarity because it does not differentiate between plants that are naturally uncommon (rare) and those that have become rare - what might be termed 'Threatened' plants. The International Union for Conservation of Nature (IUCN) makes the distinction between species that are naturally rare and those that are 'At Risk' due to external factors (Robbirt et al. 2006). The IUCN (2001) criteria for determining if a species is 'At Risk' are:

- A. Declining population, taking into account past, present and/or projected numbers.
- B. Geographic range size, and fragmentation, decline or fluctuations therein.

- C. Small population size, and fragmentation, decline or fluctuations therein.
- D. Very small population size or very restricted distribution.
- E. Quantitative analysis of extinction risk.

Rarity is a useful predictor for extinction risk because rare species – regardless of the cause of rarity - are more vulnerable to extinction (de Lange & Norton 1998; Harnik et al. 2012). Under the New Zealand Threat Classification System, species that are naturally rare are classified as 'At Risk'. If a 'Naturally Uncommon' taxa has fewer than 250 mature individuals, it shifts from 'At Risk' to qualifying as 'Threatened-Nationally Critical' (de Lange et al. 2013).

In New Zealand, a disjunct distribution is a form of rarity relating to geographic range, where populations are small but widely scattered (Wardle 1963; Rogers & Walker 2002; Shepherd & Perrie 2011; de Lange et al. 2013). Disjunct distributions may arise naturally due to a plant's long distance dispersal strategies or as a result of vicariance, the separation of populations by a biotic or abiotic factor disrupting previously linked populations. This can occur either naturally by glaciation or by anthropogenic causes such as forest clearing for agriculture (McGlone 1989; McGlone et al. 2001; Shepherd & Perrie 2011). In this thesis, I present the results of my biological study of one of New Zealand's rare plant species with a disjunct distribution, with the aim of informing its conservation management.

### **The conservation genetics of plants**

The major aim of conservation genetics is to reduce extinction risk from genetic factors (Frankham et al. 2010). These genetic factors include the negative effects of inbreeding (mating between relatives), loss of gene flow following habitat loss, and the effects of genetic drift (random changes in allele frequencies). For threatened species to persist in the long term, they need sufficient genetic variation to be able to evolve and adapt as well as large enough populations to counteract the effects of inbreeding (Gaggiotti 2003; Spielman et al. 2004; DeSalle 2005; Frankham et al. 2014).

Having a small population size, increases the effects of genetic factors such as inbreeding (Ellstrand & Elam 1993; Young et al. 1996; Honnay & Jacquemyn 2007; Frankham et al. 2014). Recessive deleterious alleles in a population are more likely to be 'hidden' in large populations. In contrast, within a small population, mating with relatives becomes more likely, and increasing homozygosity increases the chance that recessive deleterious alleles will be expressed. This is known as inbreeding depression: the decrease of fitness because of mating between relatives (Gaggiotti 2003; Frankham et al. 2010). A meta-analysis by Angeloni et al. (2011) found that inbreeding depression is one of the main factors decreasing plant population fitness and viability.

Gene flow is the exchange of genetic information between populations. For plants this occurs via the dispersal of seeds and spores (including pollen) and occasionally whole plants or parts of plants (Ellstrand & Elam 1993; Ellstrand 2014). Habitat loss and fragmentation can reduce gene flow if populations become too isolated from one another for seeds and spores to be regularly exchanged between them (Ellstrand & Elam 1993). Gene flow is generally considered beneficial because it is important for maintaining genetic connectivity and diversity and thus for preventing inbreeding depression (Ellstrand & Elam 1993). It introduces new polymorphism into a population and thereby increases population effective size, thus counteracting random genetic drift (Balloux & Lugon-Moulin 2002). However, gene flow can be detrimental for species with small population sizes as it can prevent speciation, and reduce local adaptation by introducing maladapted genotypes.

Additionally, in small populations that remain small over generations, alleles are easily lost via genetic drift, with the risk that other alleles can potentially become fixed as genetic diversity decreases. In small populations, selection is often weak in comparison to genetic drift; as genetic drift is random, deleterious alleles may become fixed by chance (Ellstrand & Elam 1993; Frankham et al. 2009).

The combined effects of random genetic drift, inbreeding and gene flow on a population can decrease the fitness of the species, resulting in reduced growth rates, lowered fecundity and increased mortality of progeny and seeds (Spielman et al. 2004; Frankham 2005). These populations are then less resilient to disturbance, fragmentation and stochastic events such as environment catastrophes (i.e. floods, fire) (Ellstrand & Elam 1993; Kramer & Havens 2009).

Many threatened species have small population sizes and reduced distribution ranges resulting from threats such as habitat loss, predation and competition from other species. One focus of conservation genetics is investigating the genetic consequences of habitat loss and the fragmentation of populations into smaller remnants where stochastic factors and inbreeding increase the risk of extinction (Frankham et al. 2009; Frankham et al. 2010). Habitat fragmentation can be defined as the reduction of larger (or continuous) tracts of habitat into many smaller, spatially isolated remnants (Young et al. 1996; Aguilar et al. 2008). Habitat fragmentation can occur in three ways: through the loss and destruction of habitat, the reduction in size of existing fragments and the further isolation of fragments (Aguilar et al. 2008). The fragmentation of habitat can lead to separation of pollinators from plants and potentially limit gene flow between once connected populations (Ellstrand & Elam 1993).

Rare plant species are often heavily affected by the loss of just a few individuals. In a small population, this can be a significant percentage of the remaining individuals that make up the population. In addition, the loss of an individual's genetic material from the gene pools can compound the effects on a rare species in the short term if significant sources of heterozygosity have been lost. In the long term,

reduction of gene flow prevents new genetic diversity being introduced to a population (Young et al. 1996; Frankham et al. 2010).

In the restoration of plant populations, careful selection of seeds or seedlings (eco-sourcing) is required to increase the level of heterozygosity while preventing loss of genetic diversity in a given population (Krauss & He 2006; Kramer & Havens 2009, Stevens et al. 2015). Conservation genetic data can help with identifying populations that are in need of restoration as well as with selecting the most appropriate donor populations for eco-sourcing. If a population has low genetic diversity, increasing the population size and gene flow may help to increase genetic diversity. In the restoration of plant populations, careful eco-sourcing is required to increase the level of heterozygosity and prevent any further loss of genetic diversity in a given population.

Improper selection of seed sources, such as the use of non-local seeds, can hinder species restoration (McKay et al 2005, Stevens et al. 2015). Firstly, introducing a novel genotype that is maladapted to the site can result in outbreeding depression and cause a decrease in population fitness because of hybridisation between genetically different individuals or populations that are the same species (Hufford & Mazer 2003; Krauss & He 2006; Frankham et al. 2010; Mijangos et al. 2015). Secondly, a 'founder effect' from collecting seed from one or very few individuals can increase the chances that a plant is surrounded by siblings and that inbreeding will occur in the population (Hufford & Mazer 2003; McKay et al. 2005). These risks are minimised in seed selection informed by genetic data.

Conservation genetic studies can also aid in prioritising the allocation of resources to conservation projects, by indicating which species and populations are most at risk of losing genetic diversity (Stevens et al. 2015). Populations of a threatened species with relatively low genetic diversity could be prioritised for active conservation efforts, while populations with higher genetic diversity may only need to be monitored for changes in population size. The use of conservation genetics is therefore becoming increasingly important to the management of rare and at risk species (Franks 2010).

Further, conservation genetics can challenge preconceived notions that may limit the effectiveness of conservation programs. For example, it was previously assumed that woody outcrossing plants were less at risk of genetic drift following habitat fragmentation due to their longer lifespan (Young et al 1996; Hamrick 2004, Lowe et al. 2005). However, this has been shown to be false in a number of populations of *Fagus sylvatica* (Jump & Penuelas 2006) and *Quercus* spp. (Sork et al. 2002, Vakkari et al. 2006; Vranckx et al. 2012), which have shown significant population divergence, reduction of genetic diversity and inbreeding when their habitats are fragmented, due to limited pollen flow between fragments (Vranckx et al. 2012). This increases the priority of remediating habitat fragmentation for such

populations, where previously this important step may not have been taken on the assumption that the gene pool of such plants would be minimally affected by the fragmentation.

### **Marker choice for conservation genetic studies**

Genetic markers are useful tools for measuring levels of genetic diversity to aid with conservation efforts of plant species. When used for diploid organisms, genetic markers are inherited characters that have one or two states (alleles) for each character (locus). Deciding which molecular marker to use is influenced by resources, time, and the exact biological questions that are being asked (Nybom 2004; Meudt & Clarke 2007). With co-dominant markers, such as microsatellites, both character states can be visualised. This allows heterozygotes to be distinguished from dominant homozygotes. With dominant markers, such as ISSRs or AFLPs, only one allele can be visualised at each locus. This means that heterozygotes cannot be distinguished from dominant homozygotes and therefore a direct measure of heterozygosity cannot be obtained. An advantage of co-dominant markers is therefore that they are more powerful than dominant markers and require smaller sampling sizes for a similar level of information (Meudt & Clarke 2007). This limitation of dominant markers can be partially overcome by giving more power to analyses by using more loci (Meudt & Clarke 2007).

Microsatellites are tandem repeated motifs of 1–6 bp and they are found throughout the genome (Kalia et al. 2011). Microsatellites are a popular marker for conservation genetic studies, as genetic variation can be assessed from only small amounts of DNA. They have co-dominant inheritance and fast mutation rates, and are often highly species-specific, which means that the risk of cross-species contamination is low. However, because sequence data are required, developing microsatellite markers is relatively expensive and has significant technical requirements (Robertson 2006; Selkoe & Toonen 2006).

The Random Amplification of Polymorphic DNAs (RAPD) is a simple method for the detection of polymorphisms based on random amplification of DNA decamers from a single arbitrary primer (Williams et al. 1990). RAPDs are a fast and cheap method of deriving information from limited amounts of DNA without *a priori* knowledge of the species genome (Hadrys et al. 1992, Perez et al. 1998). Some drawbacks of RAPDs are that they are dominant markers that have relatively low reproducibility, a high rate of non-specific amplification and are considered less informative than other methods, that is, fewer loci can be obtained from a single primer (Perez et al. 1998).

Amplified Fragment Length Polymorphism (AFLP) is a popular and commonly used dominant marker system for plant studies (Vos et al. 1995; Nybom 2004; Bensch & Akesson 2005, Meudt & Clarke 2007). AFLP is a technique that generates DNA profiles through PCR amplification of genomic DNA fragments



that are produced using restriction enzymes (Vos et al. 1995). Just like RAPDs, AFLPs do not require prior sequence knowledge. AFLP data are generally highly informative and reproducible, but are prone to contamination and need high quality DNA. This technique is notably labour and time intensive, and there can be issues with homoplasmy (co-migration of non-related amplified sequences) (Vos et al 1995). While not the most expensive marker choice, AFLPs are at least four times the cost of ISSRs (Stevens et al. 2015).

Inter-simple Sequence Repeats (ISSRs) are another dominant marker and were chosen for the present study. A single primer, based on a microsatellite sequence and potentially a short (2-4 nucleotide) anchoring sequence is used to amplify the region between closely spaced, oppositely oriented microsatellites (Zietkiewicz et al. 1994, Moreno et al. 1998). In the ISSR technique, multiple fragments are amplified, producing a characteristic pattern or genetic 'fingerprint' (Zietkiewicz et al. 1994). The ISSR technique requires no *a priori* knowledge of the genome of the species studied, and primers made of a few microsatellite repeats, such as 'AG', may be selected arbitrarily to screen a species (Bornet & Branchard 2001; Bornet et al. 2004). Alternatively, primers known to be polymorphic for the species or related species may be used. Anchoring sequences, which attach a short nucleotide sequence to either the 3' or 5' end of the primer, extend the primer length and select for amplification the chosen end of the microsatellite. Longer primers allow for higher annealing temperatures, which increase the stringency and reduce the amount of smearing on the bands. Anchoring the primer at an end of a microsatellite avoids the band smearing associated with internal priming (Blair et al. 1999; Joshi et al. 2000; Pradeep Reddy et al. 2002) .

Concerns have been raised about using the ISSR technique, because of doubts about homology, reproducibility of fingerprints and dominance of the markers (see below; Mijangos et al. 2015). However, because of their modest cost and low technical requirements, they are the marker choice for this study. ISSRs have been successfully used in conservation genetic studies of other threatened plants, both in New Zealand (Clarkson 2011; Clarkson et al. 2012; Grierson 2014) and globally (a review: Nybom 2004; see Coppi et al. 2010; Liu et al. 2012; Cires et al. 2013).

### **Genetic analyses using dominant markers**

Many techniques used in conservation genetics involve the use of dominant markers. There are, however, limitations in studying genetic diversity and genetic structure with dominant markers (such as ISSRs) compared to using co-dominant markers (such as microsatellites). Because of the nature of dominant markers, it can only be assumed that they are biallelic and that one allele is dominant and the other recessive (Holsinger 2002; Nybom 2004). When scoring fragments generated using a dominant marker system, the presence of a fragment indicates the presence of at least one copy of the dominant

allele, but does not provide further information as to whether the individual is homozygous or heterozygous for that allele (Lynch & Milligan 1994; Nybom 2004). The absence of a fragment indicates that the individual is homozygous recessive for the allele, or that the allele is not present in the individual. Dominant markers do not allow one to distinguish between these two possibilities (Meudt & Clarke 2007). In contrast to co-dominant markers, heterozygosity cannot be measured directly, but instead has to be calculated when dominant markers are used.

Expected heterozygosity is an index calculated from allele frequencies. Without knowledge of whether an individual is homozygous or heterozygous for the dominant allele, this is not easy to calculate. Therefore, heterozygosity must be inferred for analyses that require this information (Lynch & Milligan 1994; Nybom 2004). This can be done by assuming Hardy-Weinberg equilibrium when estimating allele frequencies (Bonin et al. 2007). However, this only works on the assumption that there is no inbreeding in the population, because inbreeding leads to deviations from Hardy-Weinberg equilibrium. The validity of such an assumption is species and population dependent. A lack of inbreeding is more likely to occur in species with an outcrossing mating system than one with a selfing mating system (Krauss 2000; Nybom & Bartish 2000). Heterozygosity can be calculated from dominant data using a variety of techniques, depending on the data available. The software GenAlEx calculates allele frequencies from the proportions of recessive homozygotes assuming Hardy-Weinberg equilibrium (Peakall & Smouse 2012).

When estimating allele frequencies and genetic diversity from dominant marker systems, one needs to assume that co-migration of fragments implies that they are of equal molecular weight and homologous (Lynch & Milligan 1994, Bonin et al. 2007). The assumption of homology is problematic in dominant marker studies, as non-homologous fragments are known to co-migrate, leading to allele homoplasy. Homoplasy can also occur for band absence, as fragment loss can occur from different mutations (Meudt & Clarke 2007; Simmons et al. 2007). Homoplasy reduces estimated genetic diversity, lowering the potential strength of analysis derived from dominant marker systems (Vekemans et al. 2002; Koopman & Gort 2004). However, Rieseberg (1996) found that when using RAPDs, at the intra-specific level most bands were identical by descent. This is likely to apply to other anonymous fragment-based genotyping methods, including ISSRs and AFLPs, as well.

To maximize the power of genetic analyses conducted using dominant markers, it is therefore recommended that highly polymorphic markers are used and that there is substantial sampling of individuals within populations. The issue has been debated (see Lynch & Milligan 1994; Zhivotovsky 1999), but Krauss (2000) concludes that if highly polymorphic markers are chosen, most biases are eliminated and accurate estimates of heterozygosity are produced. Krauss also recommends sampling

at least 30 individuals per population (Krauss 2000). However, this is not always possible with rare and endangered plants, because of small population size, or the life form of the plant - as in the case of small annuals, where sampling may necessitate killing the whole plant. For example, *Teucrium parvifolium* (Lamiaceae) has sites with a population of one tree (Boot 1998) and in the case of *Pittosporum obcordatum* (Pittosporaceae; the topic of this study), only four trees are known at a site in the Hedgehope catchment (Rance & Simpson 2000).

### Estimating population structure with dominant markers

Contemporary methods of genetic analysis of population structure are built on a foundation formed by the work of Wright (1965), whose  $F_{ST}$  summary statistic defined the early methods for determining population structure. Wright's  $F_{ST}$  can be defined as "the proportion of genetic diversity due to allele frequency differences among populations" (Holsinger & Weir 2009). The development of so-called F-statistics enabled researchers to assess genetic variation between populations and to develop theoretical models of migration, demographics and evolution (Wright 1965). These early statistical techniques do not necessarily work well with regard to natural populations, as the assumptions of Hardy-Weinberg equilibrium and that all populations are of the same size are often violated. However, later developments for more accurate genetic differentiation of populations have been built upon Wright's model. Cockerham (1973) redefined Wright's  $F_{ST}$  as a ratio of genetic variances to be used with multiallelic loci.

Analysis of molecular variance (AMOVA) is a statistical method that was developed to take into account new, more variable genetic markers (Excoffier et al. 1992). The AMOVA method computes  $F_{st}$  or analogues ( $\Phi_{ST}$ ,  $\Phi'_{ST}$ ) using the analysis of variance (ANOVA) approach against a matrix of squared Euclidean distance between DNA haplotypes, essentially creating ratios of variation between populations in relation to variation over all populations. The  $F_{ST}$  analogue  $\Phi_{ST}$  is appropriate for use with dominant data (Holsinger & Weir 2009). When this method is used with dominant marker data,  $\Phi_{ST}$  is bounded between 1 (complete differentiation between populations) and 0 (no differentiation between populations). As variation between populations often depresses potential overall variation, a standardised function of  $\Phi_{ST} - \Phi'_{ST}$  - has been proposed. The function  $\Phi'_{ST}$  is derived by dividing  $\Phi_{ST}$  by the maximum possible  $\Phi_{ST}$  value ( $\Phi'_{ST} = \Phi_{ST} / \Phi_{STmax}$ ). The value of  $\Phi'_{ST}$  for the analysis of population structure is that it is independent of the marker system used to gather the data, and thus allows comparison between studies done with different marker systems or effective population sizes (Meirmans 2006; Meirman & Hedricks 2011).

## Cluster analysis

STRUCTURE is a software package commonly used in population genetics for Bayesian cluster analyses for genotype assignment and detecting migrants (Pritchard et al. 2000). STRUCTURE aims to cluster individuals in the data set into one of a number of genetic clusters (K), with the intention of finding the number of clusters ( $K=n$ ) with the highest probability from a set of possible K values chosen by the researcher (Pritchard et al. 2000). For example, the researcher may select K as being potentially between one and ten, with STRUCTURE identifying K=6 having the highest probability. For each value of K, STRUCTURE determines the probability of an individual belonging to a cluster (Pritchard et al. 2000). It uses a Monte Carlo Markov Chain (MCMC) algorithm to propose potential groupings of individuals into clusters (Pritchard et al. 2000; Falush et al. 2003). The proposed allele frequency of each proposed cluster is then compared to the real allele frequency of the individuals assigned to each proposed cluster, generating a probability value that the number of clusters and individual assignment are the true assignment of clusters. The initial proposed clustering is selected arbitrarily from the parameter values, and proposed clusters are then iteratively generated, with parameter values being updated to move the algorithm towards areas of higher probability (Bonin et al. 2007). Due to this initial arbitrary clustering, a selection of the early iterations are ignored by the program as a 'burn-in' period, to avoid this biasing the later iterations affecting the overall result (Falush et al. 2007). Falush et al. (2007) developed a model to allow for the analysis of dominant data, by adding an additional step to STRUCTURE's algorithm so it no longer assumes that individual genotypes are known. Instead, for each iteration of the algorithm the diploid genotypes are updated based upon the probabilities of all possible genotypes.

### **Study species: *Pittosporum obcordatum* Raoul. (Pittosporaceae)**

Pittosporaceae is a family composed of nine genera and approximately 240 species, of which *Pittosporum* is both the type genus and largest genus (Cayzer et al. 2000; Wood & Kiehn 2011; Clarkson et al. 2012). Pittosporaceae is a family with high levels of endemism and few widespread, common species (Chandler et al. 2007). Pittosporaceae is largely confined to Australia, with *Pittosporum* the only genus with species present in other parts of the world. *Pittosporum* species are found throughout the Pacific, Asia and Africa (Gemmill et al. 2002). A study in the 1950s noted 12 species of *Pittosporum* occurring in Australia and 18-26 species occurring in New Zealand (Cooper 1956).

There is marked hybridisation among New Zealand's *Pittosporum* species, including between divaricating and non-divaricating species (e.g. *P. turneri* x *P. divaricatum*; Carrodus 2009). This suggests that there are weak reproductive barriers between species, which has complicated efforts to

determine the number of species in New Zealand (Clarkson & Clarkson 1994; Carrodus 2009). Recent studies place the number of New Zealand *Pittosporum* species at 21, all of which are considered endemic (de Lange et al. 2006; Clarkson et al. 2012). Species of the *Pittosporum* genus are found on all three of New Zealand's main islands, although many of the species have restricted ranges (de Lange et al. 2006). *Pittosporum* species are distinguished from other species in the family Pittosporaceae by having unilocular fruit, with the seed suspended in viscid pulp (Cayzer et al. 2000).

*Pittosporum obcordatum* Raoul (Pittosporaceae) is one of New Zealand's species of *Pittosporum*. It was first described by E.F.L. Raoul, a French naval doctor stationed in Akaroa on Banks Peninsula in the early 1840s (Raoul 1844; Simpson 1976). *Pittosporum obcordatum* was known earlier to Māori as 'kōhūhū' and was used medicinally for treating skin conditions, such as scabies (Goldie 1905). The small, typically heart shaped leaves of *P. obcordatum* provide its common name: 'heart leaved kōhūhū'. *Pittosporum obcordatum* exhibits heteroblasty, with juvenile leaves morphologically different from adult leaves (Cooper 1956; Clarkson & Clarkson 1994). It is a small tree or shrub and does not grow taller than c. 10 m. It typically forms a single trunked columnar tree 5-8 m in height, with notably slender, interlacing and divaricating branches (Cooper 1956; Clarkson & Clarkson 1994).

Populations of *P. obcordatum* flower at different times within the spring-early summer period, depending on location. The Kaitia population flowers as early as mid-October, whereas populations that grow at higher elevation and at more southerly latitudes flower as late as December (Clarkson & Clarkson 1994). *Pittosporum obcordatum* is dioecious and thus its flowers are unisexual and occur on separate plants (Cooper 1956). They are pink maroon or pale yellow with highlights of red on the margins or striping, and produce fragrance at night suggesting insect (moth) pollination (Clarkson & Clarkson 1994). *Pittosporum obcordatum* is assumed to be entomophilous; honeybees have been seen visiting flowers at Ngatapa and Waikohu (Clarkson & Clarkson 1994; Webb et al. 1999). However, Kelly et al. (2010) notes that birds visit flowers of other *Pittosporum* spp. and that New Zealand birds visit many entomophilous flowers.

*Pittosporum obcordatum* has a predominantly outcrossing mating system and an approximately equal sex ratio of individuals, with the addition of 6.66% of male plants producing small amounts of seed (Clarkson & Clarkson 1994). The capsules produced by *P. obcordatum* are bivalved and the fruit follows the *Pittosporum* pattern of being unilocular and having seeds immersed in viscid pulp (Cooper 1956; Clarkson & Clarkson 1994). The mode of dispersal of *P. obcordatum* seeds is poorly studied. However, it is likely to be bird dispersed, similar to the other *Pittosporum* species (Burrows 1994; Clarkson & Clarkson 1994; Gemmil et al. 2002).

There has been debate about whether an infraspecific classification of *Pittosporum* into two taxonomic varieties is justified. Laing and Gourlay (1935) proposed distinguishing *P. obcordatum* var. *kaitaiaensis* from the typical variety. Variety *kaitaiaensis* is now presumed to only occur in cultivation. This proposed classification was based on morphological features. The Kaitaia variety was described as having larger and hairier juvenile leaves, and adult leaves that are predominantly linear, as opposed to heart shaped. Clarkson and Clarkson (1994) thought that the taxonomic recognition of var. *kaitaiaensis* was unwarranted because they thought these morphological differences could be attributed to a cline. *Pittosporum obcordatum* was included in a study by Carrodus (2009) on hybridization. This study concluded that *P. obcordatum* evolved its divaricating morphology independently from other divaricating *Pittosporum* (*P. turneri*, *P. divaricatum*, *P. anomalum* and *P. rigidum*). It also found that *P. obcordatum* was placed in a clade that is distinct from the main New Zealand clade (Carrodus 2009).

*Pittosporum obcordatum* has a disjunct distribution of uncertain origin and widely separated populations with unknown genetic diversity. It has a discontinuous lowland distribution with populations ranging from Northland to Southland (Figure 1.1), but with a tendency to occur on the eastern side of New Zealand in lowland forest habitats <500 m (Clarkson & Clarkson 1994).

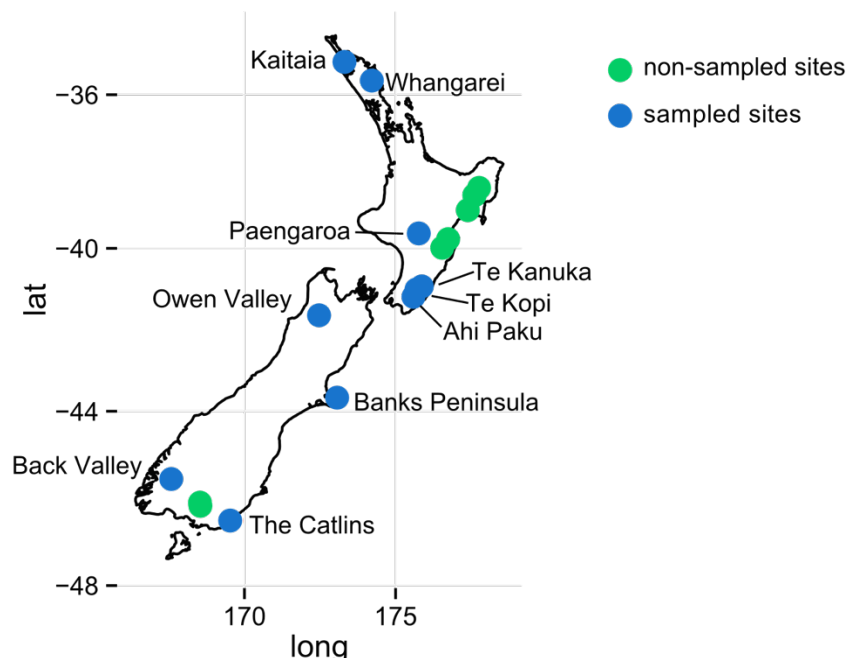


Figure 1.1 The distribution and the sampling locations used in this study of *P. obcordatum*.

Many of New Zealand's *Pittosporum* species are of conservation concern, as of the 21 known species, five are considered 'Threatened' and another six are classified as 'At Risk' (de Lange et al. 2009). In the most current list of threatened New Zealand plants, *P. obcordatum* has been listed in the category of 'Threatened' with a conservation status of 'Nationally Vulnerable' (de Lange et al. 2013). This listing is based on the evaluation that there are only 1000-5000 mature individuals of *P. obcordatum* in the wild and that the total number of populations and total population size have probably decreased through habitat clearance (de Lange et al. 2013). There have been various estimates of the number of *P. obcordatum* individuals in the wild, as the species history is a catalogue of populations being found, lost and refound (Given 1981, King 1986, Clarkson & Clarkson 1994). Clarkson and Clarkson (1994) estimated that there are fewer than 2500 wild individuals of *P. obcordatum*. Based on recent discoveries of new populations at Owen Valley (2009), Banks Peninsula (2012) and Whangarei (2014), and thorough counts, the current estimate (following the studies carried out for this thesis) of *P. obcordatum* in the wild is ~1,985 (Table 1.1).

The threats to *P. obcordatum* include habitat loss and fragmentation, a lack of recruitment of seedlings, grazing from introduced mammals, competition from exotic plants and changes in hydrological regimes (Clarkson & Clarkson 1994; de Lange et al. 2010; Hutchison 2014). Small population size is another concern for the long-term persistence of *P. obcordatum* populations. There are only two populations with more than 200 individuals, with most of the remaining populations having less than 100 individuals (Clarkson & Clarkson 1994; Hutchison 2014).

The rarity of *P. obcordatum* has been previously attributed to habitat specialisation combined with habitat loss and fragmentation (Clarkson & Clarkson 1994). The specific habitat of *P. obcordatum* is lowland alluvial flats, with primary, secondary or mixed shrub forest on fertile base rich soils. Such forests were once widespread in New Zealand, but because of their accessibility and fertility these were largely cleared for human use (Norton & Miller 2000). Clarkson & Clarkson (1994) described it as a very linear habitat, mainly on river flats, usually near swamps, oxbow lakes, sometimes on colluvial toeslopes but never on hillslopes. Observation of the Southland populations of *P. obcordatum* has suggested that these populations have potentially adapted to a flooding regime (Clarkson & Clarkson 1994; Walker et al. 2006). *Pittosporum obcordatum* has been observed establishing new cohorts in areas where canopy gaps are caused by flooding. It also takes advantage of dieback in species that are less tolerant of flooding (Walker et al. 2006). Some authors have even suggested *P. obcordatum* is so reliant on flooding in order to occupy a given area (Clarkson & Clarkson 1994). In this case, continued flooding could be an indicator for the health of these habitats. Our current knowledge of *P. obcordatum* indicates that it tends to co-occur in habitats alongside divaricating or other small-leaved shrubs. For example, these communities often include species such as *Coprosma pedicellata*, *Myrsine divaricata*,

*Melicytus flexuosus* and *Sophora microphylla* (King 1986; Clarkson & Clarkson 1994; Rance & Simpson 2000; Rance 2007).

### **Study sites**

Field work for this research project encompassed visiting nine sites across the length of New Zealand (Figure 1.1). In addition, samples from Te Kopi (Wairarapa) were collected by Department of Conservation (DOC) staff. Sites were chosen based on DOC records, the New Zealand Plant Conservation Network (NZPCN) database and access to private land (Table 1.1). The Owen Valley site is the only locality that occurs west of the main divide. In the South Island, all sites are forest fragments on farms, apart from the Back Valley site, which occurs in Fiordland National Park and has never been grazed or cleared. All other South Island sites have been modified in some way by human land use. Owen Valley and the Catlins location are fenced off to exclude mammals, while the Banks Peninsula site has no fenced areas. Six sites were visited in the North Island. The two sites visited in the Wairarapa, Ahi Paku and Te Kanuka, are both fenced to exclude grazing mammals. Ahi Paku is protected by a Queen Elizabeth II (QEII) open space covenant, but Te Kanuka currently has no legal protection. Paengaroa (DOC), Whangarei (DOC, council and private) and Kaitaia (QEII) all have legal protection and some form of fencing.



Table 1.1 Habitat descriptions of *P. obcordatum* sites sampled. Showing site name, longitude, latitude, herbarium voucher, census size and threats.

| Site            | Longitude, latitude.   | Voucher number | Census size        | Habitat description  | Threats  |
|-----------------|------------------------|----------------|--------------------|--|--|
| Back Valley     | -45.589773, 167.57833  | saw29          | ~700 <sup>1</sup>  | Intact, mixed shrubland.<br><br>DOC National Park              | Wild deer  |
| The Catlins     | -46.542547, 169.528768 | saw24          | ~8 <sup>2</sup>    | Lowland remnant forest on private land                         | Competition exotic plants, population size           |
| Banks Peninsula | -43.701595, 173.066401 | saw2           | 65 <sup>3</sup>    | Hillslope, podocarp-hardwood on private land                   | Grazing, competition, exotic grass, population size  |
| Owen Valley     | -41.674579, 172.469017 | saw8           | 23 <sup>4</sup>    | Lowland, podocarp-hardwood on private land                     | Competition exotic plants, population size.          |
| Te Kopi         | -41.215014, 175.592266 | No voucher     | 7 <sup>5</sup>     | Remnant on private land  | Competition exotic grass, forestry, population size. |
| Ahi Paku        | -40.957474, 175.867632 | saw55          | 60 <sup>5</sup>    | Mixed shrubland-podocarp covenant on private land              | Population size                                      |
| Te Kanuka       | -41.034305, 175.691192 | saw63          | 50-60 <sup>5</sup> | Fenced swamp terrace on private land                           | Population size                                      |
| Paengaroa       | -39.620735, 175.768863 | saw73          | 20-30 <sup>6</sup> | Mainland island scenic reserve & railway land. Mixed shrubland | Cattle, competition exotic plants, population size   |
| Whangarei       | -35.619419, 174.215305 | saw88          | 734 <sup>7</sup>   | Hikurangi Swamp/Wairua reserve                                 | Competition exotic plants                            |
| Kaitaia         | -35.117522, 173.309524 | saw108         | 7 <sup>7</sup>     | Covenant on private land. Kahikatea forest                     | Competition exotic grass, population size            |

Note: Census citations, <sup>1</sup> Rance et al. 2007; <sup>2</sup> J. Barkla pers. comm.; <sup>3</sup> Hutchison 2014; <sup>4</sup> Sandra Wotherspoon pers. comm.; <sup>5</sup> T. Silbery pers. comm.; <sup>6</sup> C. Ogle pers. comm.; <sup>7</sup> A. Townsend pers. comm. Non-sampled population estimates: Southland 25 (Rance & Simpson 2000); Hawkes Bay 266 (Walls 1998).

## Outline of thesis

The overarching goal of this thesis was to better understand genetic and ecological factors that could assist with the conservation *P. obcordatum*, a threatened New Zealand plant. Chapter 2 is a research chapter on the conservation genetics of *P. obcordatum*. This is the first population genetics study of *P. obcordatum*, and I use Inter-Simple Sequence Repeat (ISSR) markers to quantify genetic diversity within and between populations. I use this genetic information to propose conservation strategies for the management of all sampled *P. obcordatum* populations. In Chapter 3, I examine why *P. obcordatum* grows in an atypical hillside habitat on Banks Peninsula. I test the hypothesis that plants of *P. obcordatum* in Banks Peninsula can survive on a hillslope because they have adapted to drier conditions. I use seedlings from the Back Valley population as a point of reference. I also test the hypothesis that despite differences in landform habitat, the soil at the Banks Peninsula site is of similar fertility to those of an alluvial flat. In Chapter 4, I summarise key findings of the two data chapters and make recommendations for the conservation management and future work.

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## Chapter 2: Conservation genetics of the threatened New Zealand endemic *Pittosporum obcordatum* (Pittosporaceae)

(Note to reviewers: this Chapter is intended as a manuscript that can be easily modified for publication. It therefore contains some topics that are also addressed in Chapter 1).

### Introduction

Approximately 38% of New Zealand's native vascular flora is threatened or naturally rare (de Lange et al. 2009). The primary threat to the New Zealand flora is human modification of habitat, including habitat loss and the effects of introduced plant and animal species (Myers et al. 2000; Norton & Miller 2000). Lowland habitats (<500m) in particular have been encroached upon by human activity. Approximately 30% of New Zealand's threatened plant species only occur in lowland alluvial forests. Forty-nine percent of areas above 500m are protected, compared with 18% of land at lower elevations (Norton & Miller 2000). Furthermore, because of their economic value, New Zealand's lowlands have been largely cleared of native forest and converted for agricultural production (Green & Clarkson 2005; MacLeod & Moller 2006). This has resulted in a fragmented landscape of small pockets of natural habitat amidst a vast agricultural landscape.

A noted feature of the New Zealand flora is the current disjunct distribution of many of its species (McGlone et al. 2001; McGlone et al. 2010). These species have widely separated populations with large gaps along their distribution. The origin of these distributions is not always clear, especially when we only have a biased representation of their potential range, rather than knowledge of their pre-human settlement range (Monks & Burrows 2014). Historically, such distributions have been attributed to long distance dispersal or habitat loss caused by glaciation, for example in taxa such as *Aciphylla leighii* and *Pseudopanax ferox* (Wardle 1988; McGlone et al. 2010; Shepherd & Perrie 2011). Other species display this disjunct pattern due to presumed anthropogenic habitat loss, for example *Veronica speciosa* (Armstrong & de Lange 2005). However, because of anthropogenic habitat loss it is sometimes difficult to know whether a species naturally has a disjunct distribution pattern, or if it displays this pattern due to habitat loss resulting from human land use.

This research project focused on the conservation genetics of *Pittosporum obcordatum* Raoul (Pittosporaceae), a rare endemic New Zealand plant species with a discontinuous and highly disjunct lowland (<500 m) distribution. It was first described by E.F.L. Raoul, a French naval doctor stationed in Akaroa on Banks Peninsula in the early 1840s (Raoul 1844; Simpson 1976).

*Pittosporum obcordatum* was known earlier to Māori as ‘kōhūhū’ and was used medicinally for treating skin conditions (Goldie 1905). The small, typically heart-shaped leaves of *P. obcordatum* provide its common name, ‘heart leaved kōhūhū’ (Figure 2.1). There has been debate about whether an intraspecific classification into two taxonomic varieties is justified. Laing and Gourlay (1935) proposed distinguishing *P. obcordatum* var. *kaitaiaensis* from the typical variety. Cooper (1956) and Clarkson and Clarkson (1994) thought that the taxonomic recognition of var. *kaitaiaensis* was unwarranted because its differences were slight and could be attributed to a cline. *Pittosporum obcordatum* is a small-leaved divaricating shrub, with a dioecious mating system (Cooper 1956; Clarkson & Clarkson 1994). It is known from about twenty locations across the North and South Islands of New Zealand (Clarkson & Clarkson 1994; Table 2.1). The population size of *P. obcordatum* ranges from 6 to 734 individuals, with all but two populations having fewer than 100 individuals (Clarkson & Clarkson 1994, Rance 2007; A. Townsend pers. comm.).



Figure 2.1 Male flowers of *P. obcordatum* at Banks Peninsula (photo by Melissa Hutchison, October 2013).

*Pittosporum obcordatum* is a species that has been affected heavily by habitat loss and this may be an explanation for the small sizes of some of the populations (Table 2.1). The current distribution of *P. obcordatum* is considered unnatural (Clarkson & Clarkson 1994; de Lange et al. 2013), with Clarkson & Clarkson (1994) attributing this to its specific habitat type being largely cleared for agriculture. Threats to the species’ survival include habitat loss and modification, competition and herbivory from introduced species, and changes to disturbance regimes (Clarkson & Clarkson 1994; de Lange et al. 2010; de Lange et al. 2013; Hutchinson 2014). *Pittosporum obcordatum* has a conservation ranking of ‘Nationally Vulnerable’, based on its total population of 1000-5000 individuals in partial decline (de Lange et al. 2013; Table 1.1, Chapter 1).

Conservation genetics is increasingly recognised as an important tool in the management of threatened species (Franks 2010). Ensuring threatened species have sufficient genetic diversity helps preserve their evolutionary potential, so that they are able to adapt to future changes in their environment. High levels of genetic variation are thought to be vital for the long-term maintenance of threatened species (Frankham 2005). If small populations remain small in the long term, it can lead to negative genetic effects such as genetic drift (including genetic bottlenecks and founder events) and inbreeding. The consequences of this can be inbreeding depression, loss of allelic variation, fixation of deleterious alleles, and a resulting decrease in fitness of the population (Frankham 2005; Frankham et al. 2009).

Characteristics of threatened plant species include small population size and geographic isolation. *Pittosporum obcordatum* has these characteristics, with most populations having <100 individuals and the majority of populations being widely separated (Appendix I). Additionally, the effective population size of *P. obcordatum* is halved because of its dioecious mating system (Cooper 1956; Frankham et al. 2014). In order to maximise the evolutionary potential of *P. obcordatum*, restoring the size of these small populations would be ideal. Conservation genetic data can be used to inform conservation of these small populations by identifying populations with low genetic diversity and assessing if nearby populations may be similar enough for the eco-sourcing of seeds (Stevens et al. 2015).

While there have been many ecological studies conducted on *P. obcordatum* (King 1986, Clarkson 1991, Clarkson & Clarkson 1994, Walker et al. 2006), there has been no conservation genetics research thus far on this species. This study uses Inter-Simple Sequence Repeat markers (ISSR) to quantify the genetic variation within and between ten populations of *P. obcordatum*. ISSR markers have been used to study the genetic variation of other endangered or endemic species (e.g. George et al. 2009; Clarkson et al. 2012; Liu et al. 2012; Cires et al. 2013; Xing et al. 2015).

The overarching objective of this study was to quantify current levels of genetic diversity within and between populations of *P. obcordatum* in order to contribute to future conservation management of this species. Furthermore, I asked the following questions:

- 1) Is there a correlation between the population size of *P. obcordatum* and genetic diversity?
- 2) Is there evidence for genetic connectivity (gene flow) between populations of *P. obcordatum*?
- 3) How many genetic populations of *P. obcordatum* are there?
- 4) Is there evidence of isolation by distance between populations of *P. obcordatum*?



## Materials and Methods

### Sample collection

Sample collection was carried out in conjunction with the Department of Conservation between December 2013 and February 2014. A total of 128 leaf tissue samples were collected from ten populations (sites) of *P. obcordatum* throughout its range (Table 2.1 & Fig. 2.1). Fifteen to twenty *P. obcordatum* leaves per plant were collected from as many mature individuals as could be found in each of the ten sampled populations. Voucher specimens from one or two trees were collected at each location and lodged at the University of Canterbury herbarium (CANU, see Chapter 1). Leaf tissue was collected into silica gel in zip lock bags and subsequently stored in a -20C freezer at the University of Canterbury.

Table 2.1 Information about the populations of *Pittosporum obcordatum* sampled, including location, number of individuals per populations (<sup>e</sup>, estimated; <sup>c</sup>, counted), number of private alleles, expected heterozygosity ( $H_e$ ), and percentage of polymorphic loci (P). Refer to Chapter 1 for census sizes.

| Site                | Site number | # inds. sampled | Census size        | # private alleles | $H_e$ (standard error) | P     |
|---------------------|-------------|-----------------|--------------------|-------------------|------------------------|-------|
| <b>NORTH ISLAND</b> |             |                 |                    |                   |                        |       |
| Te Kopi             | 5           | 4               | 7 <sup>c</sup>     | 0                 | 0.086<br>(± 0.014)     | 22.78 |
| Ahi Paku            | 6           | 8               | 60 <sup>c</sup>    | 0                 | 0.142<br>(± 0.013)     | 40.51 |
| Te Kanuka           | 7           | 9               | 50-60 <sup>e</sup> | 0                 | 0.083<br>(± 0.012)     | 22.78 |
| Paengaroa           | 8           | 15              | 20-30 <sup>e</sup> | 1                 | 0.169<br>(± 0.016)     | 53.80 |
| Whangarei           | 9           | 20              | 734 <sup>c</sup>   | 0                 | 0.201<br>(± 0.016)     | 62.88 |
| Kaitaia             | 10          | 6               | 7 <sup>c</sup>     | 1                 | 0.142<br>(± 0.016)     | 37.34 |
| <b>Mean</b>         |             |                 |                    |                   | 0.137                  | 40.02 |
| <b>SOUTH ISLAND</b> |             |                 |                    |                   |                        |       |
| Back Valley         | 1           | 22              | 700 <sup>e</sup>   | 0                 | 0.157<br>(± 0.016)     | 46.30 |
| The Catlins         | 2           | 6               | 8 <sup>c</sup>     | 0                 | 0.151<br>(± 0.018)     | 35.44 |
| Banks Peninsula     | 3           | 24              | 65 <sup>c</sup>    | 4                 | 0.192<br>(± 0.018)     | 61.39 |
| Owen Valley         | 4           | 14              | 22 <sup>c</sup>    | 3                 | 0.168<br>(± 0.016)     | 39.87 |
| <b>Mean</b>         |             |                 |                    |                   | 0.167                  | 45.75 |
| <b>Overall Mean</b> |             |                 |                    |                   | 0.149<br>(± 0.005)     | 44.28 |

## **DNA extraction**

A modified CTAB method for DNA extraction was used for this experiment (Doyle & Doyle 1987). Leaf tissue was homogenised in a 1.7 mL Eppendorf tube with two sterile metal beads in a Retsch Mixer Mill MM400 (Appendix 1) for three minutes. Quantity and quality of the DNA was measured using a NanoDrop™ spectrophotometer for the first sixty samples from which DNA was extracted, but because these extractions resulted in similar DNA quality and quantity (100-200ng/uL), DNA quantity and quality were not measured for the remainder of samples. DNA was diluted to a 1:100 concentration with nuclease-free water and then used for downstream analyses. This DNA concentration resulted most consistently in successful PCR amplification.

## **ISSR PCR**

Twenty-five ISSR primers (Invitrogen) selected from those used by Clarkson (2011), Clarkson et al. (2012) and Mendes et al. (2011), were initially screened for amplification success, reproducibility of results, and levels of polymorphism using the methodology outlined below. DNA extractions from nine individuals were used for this pilot study: two from Banks Peninsula, two from Back Valley, one from Paengaroa, two from Whangarei, and two from Kaitaia. In order to evaluate the reproducibility of fragment patterns, a further four to five individuals from each population were selected for PCR replication for each primer (see below; Figure 2.2). These individuals were later used to calculate error rates. Ten primers were selected for further analysis because they amplified reliably across all individuals, provided fragment patterns with good reproducibility and showed polymorphism within and between populations (Table 2.2).

### ISSR PCR amplification

PCR was performed in a total volume of 15 µl in an Eppendorf thermocycler. Each reaction consisted of 1 µl of template DNA, 6.13 µl of nuclease-free water, 3 µl 5× Green GoTaq buffer (Promega), 1.2 µl 2.5mM of each dNTP, 1 µl of 25 pmol/µl ISSR primer, 2.4 µl of 25mM MgCl<sub>2</sub>, 0.15 µl of 10 mg/ml of bovine serum albumin (BSA) and 0.12 µl of GoTaq Flexi Taq (Promega). The cycling conditions were as follows: initial denaturation at 94° C for four minutes, followed by 40 cycles of denaturation at 94°C for 40 seconds, an annealing temperature of 50°C for 45 seconds, 72°C for 90 seconds, and a final extension step of 72°C for 5 minutes and then held

at 4°C. One negative control (mastermix without DNA template) and two positive controls (samples that amplified in previous ISSR PCRs) were included in every PCR run.

Table 2.2 Selected ISSR primers for this study, showing primer sequence, no of loci and number of polymorphic loci. Primer sequences were obtained from Clarkson et al. (2012) and Mendes et al. (2011).

| ISSR         | Primer<br>sequence<br>5'-3' | No. of loci | No. of polymorphic<br>loci |
|--------------|-----------------------------|-------------|----------------------------|
| ISSR 4       | (CA) <sub>6</sub> AC        | 17          | 16                         |
| ISSR 7       | (CA) <sub>6</sub> GT        | 20          | 20                         |
| ISSR 8       | (GA) <sub>6</sub> GG        | 16          | 15                         |
| ISSR 9       | (GT) <sub>6</sub> GG        | 11          | 11                         |
| ISSR 10      | (GA) <sub>7</sub> CC        | 14          | 13                         |
| ISSR 13      | (GAG) <sub>3</sub> GC       | 17          | 15                         |
| ISSR 807     | (AG) <sub>8</sub> T         | 16          | 16                         |
| ISSR 812     | (GA) <sub>8</sub> A         | 18          | 17                         |
| ISSR 827     | AC) <sub>8</sub> G          | 15          | 15                         |
| ISSR 836     | (AG) <sub>8</sub> YA        | 14          | 10                         |
| <b>Total</b> |                             | 158         | 148                        |

#### ISSR electrophoresis

PCR fragments were separated by electrophoresis on 100 ml 2% agarose gels in 1× sodium borate buffer (Brody & Kern 2004) with 7 µl SYBR<sup>®</sup> Safe. 15 µl of PCR product, including 3 µl of loading buffer, was loaded into each lane. 2 µl of Hyperladder 50bp (Bioline) was loaded into the first and last lane of every electrophoresis gel to provide a measure of fragment length. Gels were run for 5 to 6 hours at 60 V. They were subsequently photographed with UV light using a Syngene G: BOXEF2 imager (Figure 2.2).

### ISSR scoring

Fragments that were of an equal size were assumed to be homologous and were scored as either present (1) or absent (0) (Figure 2.2). Loci that were ambiguous were coded as missing data (i.e. as -1). Fragments that were repeatability dubious or too faint were not scored, and in some cases a whole locus was removed from further analyses because of inconsistent amplification (Figure 2.2). The ISSR data were compiled into a binary matrix for further analyses. Twenty replicates were selected from the pilot study to calculate the ISSR scoring error rate, based on the recommendations of Bonin et al. (2004).

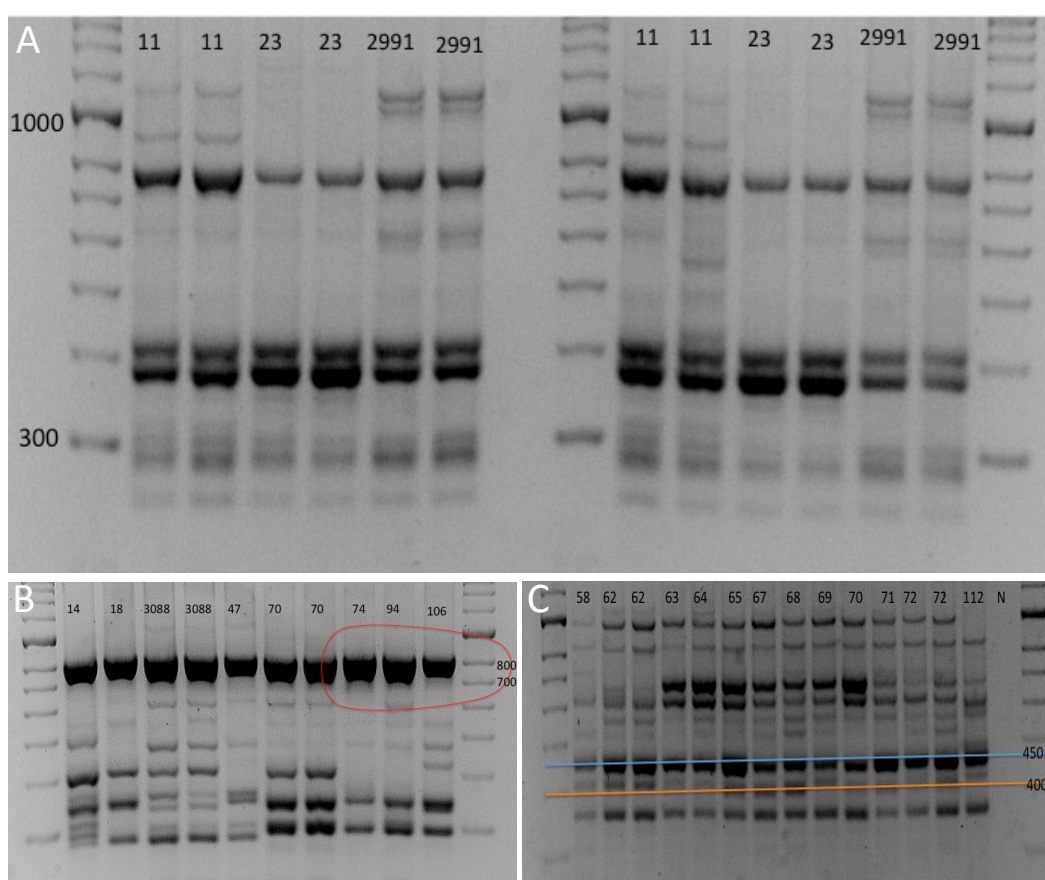


Figure 2.2 Representative gels showing fragment patterns. A. Gel of primer ISSR 812 showing replicates from two separate PCR runs. Lanes 1,9 & 16 contain Hyperladder 50 bp. Samples were from Owen Valley (11), the Catlins (23) and Banks Peninsula (2991). B. Example of ISSR 10 showing poor separation of fragments after six hours of electrophoresis. In this case, all bands at 800 bp were scored as monomorphic but any potential bands between 800 and 700bp were not scored as they did not separate completely. These bands appeared to have different molecular weights but this was not taken into consideration when scoring. C. Gel of ISSR 8 showing how bands were estimated. Samples were: 58, Ahi Paku; 62-65, Te Kanuka; 67-72, Te Kopi; 112, Kaitaia.

## Genetic analyses

### Genetic diversity within populations

Standard descriptive measures of genetic diversity were calculated from the ISSR data matrix with GenAlEx v.6.501 (Peakall & Smouse 2012). These included the percentage of polymorphic loci (P), the number of private alleles and the unbiased expected heterozygosity ( $H_e$ ) of each population (Table 2.1). To test whether population size was correlated with genetic diversity, in other words, if smaller populations have less genetic diversity, I conducted a series of Spearman's rank correlations using R version 3.1.1 (R core development team 2014). The first of these tests was to see if there was a correlation between estimated population size and P. A positive correlation could indicate that smaller populations have less genetic diversity. To ensure that the levels of genetic diversity observed were not artefacts of sampling size, another Spearman's rank between sample number per site and P was conducted. Finally, a Spearman's rank test was performed between sampling size and estimated population size, to test whether sampling size was dependent on population size. If sampling size and population size were correlated, it could mean that the levels of genetic diversity I observed could be a result of sampling an unrepresentative number of individuals.

In order to account for differences in sampling size when estimating genetic diversity, I employed the technique of rarefaction as recommended by Leburg (2002). Rarefaction was calculated with the program AFLP-div version 3.1 (Coart et al. 2005), with sampling size for each of the ten populations set to 4 (the smallest sampling size).

The adjusted value of P from the rarefaction analysis was used in a final Spearman's rank correlation between P and population size.

### Genetic structure between populations

To determine the number of genetic clusters of *P. obcordatum*, I used a Bayesian clustering method as implemented in the software package STRUCTURE v2.3.4 (Pritchard et al. 2000; Falush et al. 2003; Falush et al. 2007). STRUCTURE uses a model-based approach that employs a Markov Chain Monte Carlo algorithm to assign individuals to K genetic clusters without prior population information. Analyses were run using correlated allele frequencies and the admixture model with a burn in of 200,000 and a MCMC of 500,000 generations with 12 iterations. Because samples were collected from ten populations, K was tested for the range of 1 to 10. In this study the term 'cluster' is used to refer to genetic population.

The STRUCTURE results were uploaded to STRUCTURE HARVESTER to find the most probable value of K (Earl & VonHoldt 2012). Assigned estimates of K were visualised in STRUCTURE HARVESTER using the L(K) and  $\Delta K$  methods (Evanno et al. 2005; Earl & VonHoldt 2012). Results of the estimated K were combined and visualised into summary bar plots via the online program CLUMPAK (Cluster Markov Packager Across K) (Kopelman et al. 2015), which finds the most likely assignment for each individual based on the ten iterations.

To identify migrant individuals, I used the USEPOPINFO model in STRUCTURE. To determine whether individuals were immigrants to their populations or had recent immigrant ancestors, GENSBACK was set at three (GENSBACK=3), which evaluated immigration up to the great-grandparent level. As most individuals were assumed to be non-migrants, MIGRPRIOR was set at 0.001.

An Analysis of Molecular Variance (AMOVA) in GenAlEx was used to determine how genetic diversity was partitioned within and between populations. Populations for the AMOVA were sampled sites. For dominant data, GenAlEx calculates  $\Phi_{ST}$ ,  $\Phi'_{ST}$  and pairwise  $\Phi_{ST}$  (Peakall and Smouse 2012).  $\Phi'_{ST}$  ( $\Phi'_{ST} = \Phi_{ST} / \Phi_{ST \text{ max}}$ ) is an additional unbiased parameter that takes into account that within-population variation can be as high as total variation (Meirmans 2006). Isolation by distance was assessed using a Mantel test between geographic distances (Appendix I) and pairwise  $\Phi_{ST}$  in GenAlEx with 9999 permutations. A Bonferroni method (B-Y method) was implemented to correct for multiple comparisons for pairwise  $\Phi_{ST}$  values (Narum 2006). The results ( $P$ ,  $H_e$  and  $\Phi_{ST}$ ) for *P. obcordatum* were then compared to other ISSR studies of species that were predominantly outcrossing and are rare or have fragmented distributions.

## Results

### Genetic diversity within populations

The ISSR fragments that were scored ranged from 200 to 1200 bp (Figure 2.2). Between 11 and 20 bands were scored for each of the ten primers, resulting in a data matrix with 158 fragments scored for 128 individuals from 10 populations (Table 2.2). The rate for scoring error per primer ranged from 0.4% to 10.6%, with a mean error rate of 6.4%. At the species level, ISSR markers used in this study had a P of 94% for *P. obcordatum* (148 bands out of 158 were polymorphic).

Across all ten populations there was a mean P of 44.28%. The percentage of polymorphic loci ranged from 22.78% to 62.88% per population. The mean expected heterozygosity ( $H_e$ ) was 0.146 (Table 2.1). Expected heterozygosity ranged from 0.083 to 0.201. The Te Kanuka (n=9) and Te Kopi (n=4) populations were the least genetically diverse in terms of P and  $H_e$ . The Whangarei (n=20) and Banks Peninsula (n=24) populations were the two most genetically diverse as measured by P and  $H_e$  (Table 2.1).

Private alleles were detected in four of the populations, with more private alleles occurring in the South Island than in the North Island. The Banks Peninsula population had four private alleles, whereas the Owen Valley population had three. The Paengaroa and Kaitaia populations in the North Island had one private allele each (Table 2.1).

There were significant positive correlations for the initial Spearman's rank tests conducted between P and sampling size, P and population size, and sampling size and population size ( $r=0.77-0.82$ ,  $p\text{-value} < 0.05$ , Figure 2.3 A-C). When P was adjusted by the rarefaction technique for small sampling size, the correlation between population size and PPL was statistically significant ( $R\ 0.84$   $p\text{-value}\ 0.004$ , Figure 2.3D).

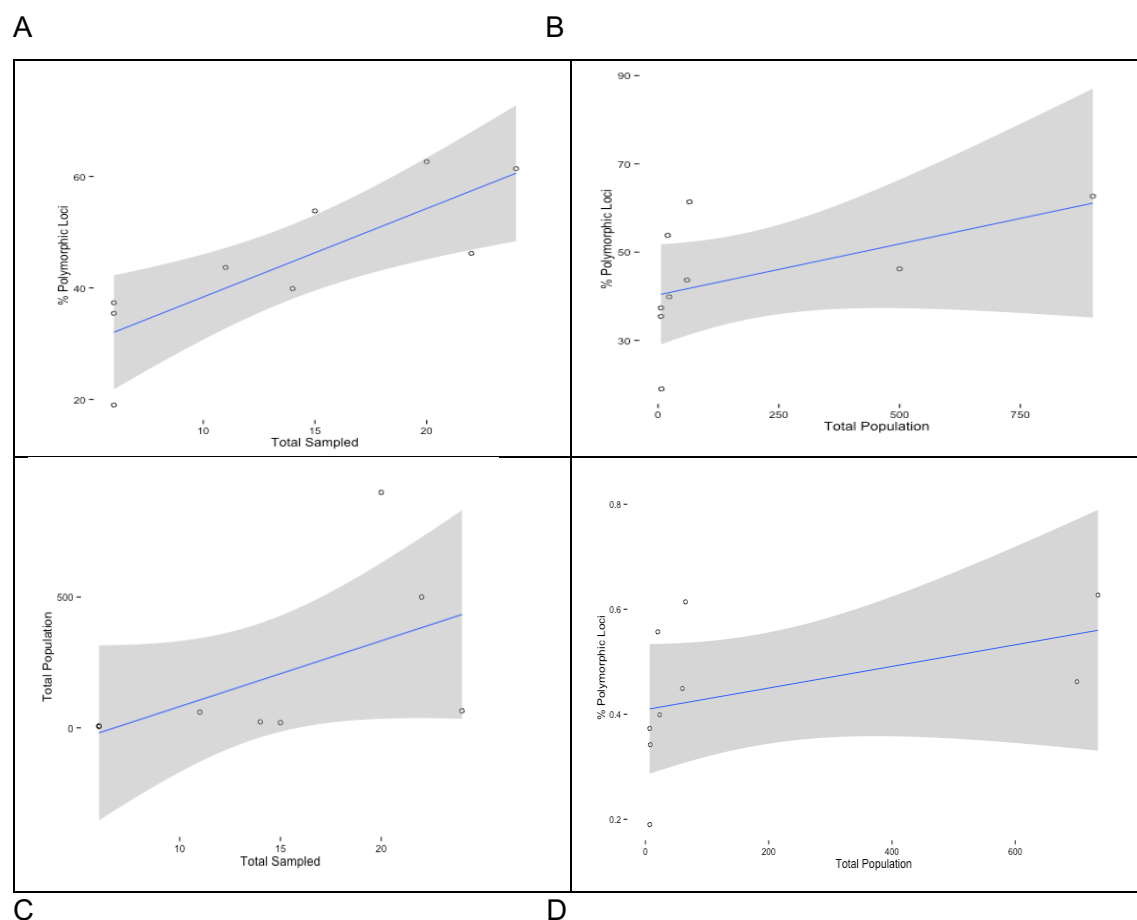


Figure 2.3 Spearman's rank correlation analyses. A. Percentage of polymorphic loci (P) vs. sampling size  $R = 0.77$ ,  $p\text{-value} = 0.02$ ; B. P and population size  $R = 0.81$   $p\text{-value} = 0.007$ ; C. Population size and sampling size  $R = 0.82$   $p\text{-value} = 0.01$ ; D. Rarefaction estimates of P vs. population size  $R = 0.84$   $p\text{-value} = 0.004$ . Grey areas represent the confidence intervals.

## Genetic structure between populations

The Evanno method of  $\Delta K$  showed support for  $K=9$  being most probable, with some support for  $K=5$  (Figure 2.4). However, the  $L'(K)$  showed that the curve levelled off at  $K=5$  (Figure 2.5).

For  $K=9$ , the genetic clusters in the South Island (site no. 1-4) largely matched collection sites (Figure 2.6). In the North Island, there was evidence of two eastern North Island clusters (dark orange and light green in Fig. 2.6). The Te Kopi (5) and Ahi Paku (6) sites showed admixture while individuals from Te Kanuka (7) were assigned to one cluster. Kaitaia (10) and Paengaroa (8) were predominantly assigned to their own genetic clusters, except for a few individuals from Paengaroa which were admixed between an eastern North Island cluster (dark orange).



Whangarei (9) showed evidence of affinities with both Paengaroa and Kaitaia clusters (Figure 2.6).

For  $K=5$ , all sites were assigned to genetic clusters based on geographic proximity (Figure 2.6). In the South Island, there were three genetic clusters. The populations at Owen Valley (northern South Island) and Back Valley (Fiordland) were assigned to their own unique genetic clusters, while the Catlins and Banks Peninsula were clustered together. For  $K=5$ , the North Island had two genetic clusters, with Te Kopi, Ahi Paku and Te Kanuka forming an eastern North Island cluster. Although Paengaroa is geographically closer to the eastern North Island, it clustered with the two Northland sites (Whangarei and Kaitaia) (Figure 2.6). The results from the migrant testing in STRUCTURE showed no evidence for recent immigrants in any population.

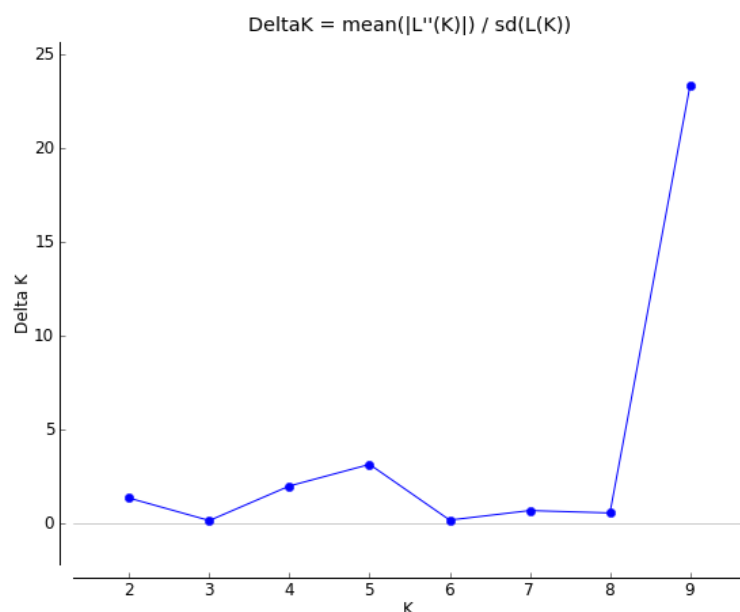


Figure 2.4  $\Delta K$  versus the number of genetic clusters (K). The average was calculated from 12 replicates of each K by the software STRUCTURE. The data was summarised by STRUCTURE HARVESTER (Earl & von Holdt 2012).

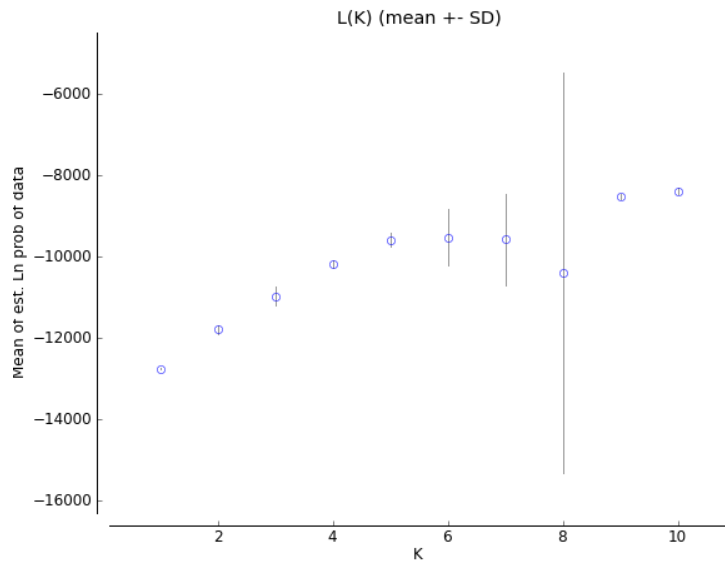


Figure 2.5 The mean likelihood  $L(K)$  and variance per  $K$  from 12 STRUCTURE runs. The data was summarised by STRUCTURE HARVESTER (Earl & von Holdt 2012).

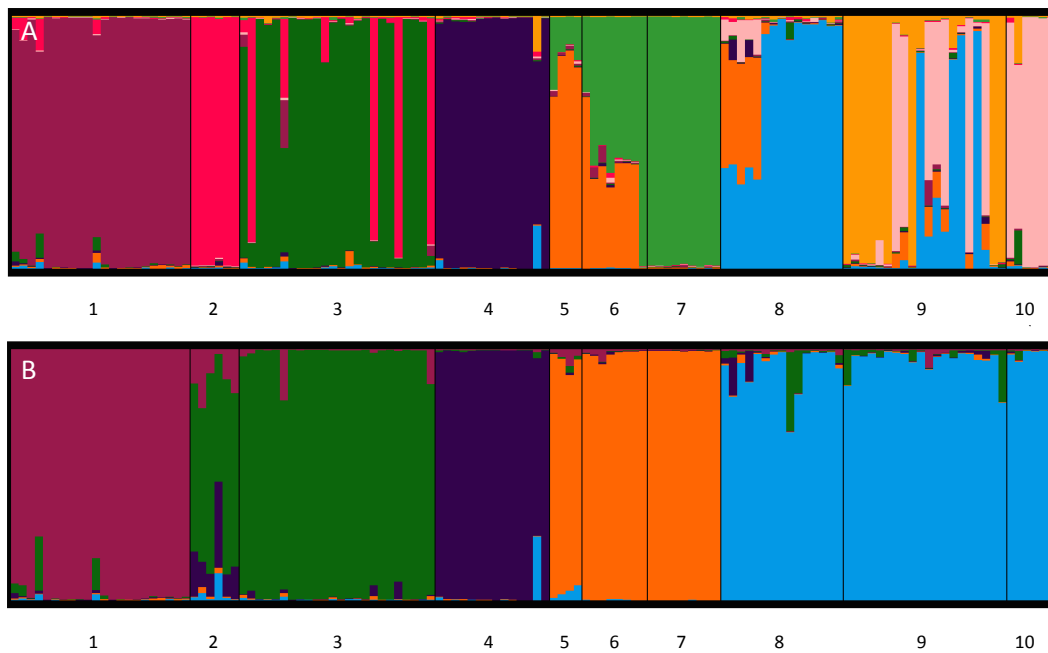


Figure 2.6 Results of STRUCTURE plots combined by CLUMPAK for 12 iterations for  $K=9$  (A) and  $K=5$  (B). Black vertical lines represent boundaries between sites. Colours indicate individuals' assignment to a genetic cluster. Sites shown are South Island 1 Back Valley, 2 The Catlins, 3 Banks Peninsula, 4 Owen Valley; North Island 5 Te Kopi, 6 Ahi Paku, 7 Te Kanuka, 8 Paengaroa, 9 Whangarei, 10 Kaitaia.

The AMOVA revealed that 44% of the genetic variation was partitioned between populations, with 56% found within populations (Table 2.3). The overall  $\Phi_{ST}$  for *P. obcordatum* was 0.44 ( $P < 0.001$ ) and the  $\Phi'_{ST}$  value which corrected for within-population variation was 0.532. Pairwise  $\Phi_{ST}$  values ranged from 0.168 to 0.650 (Table 2.4). All 45 pairwise comparisons were significant after Bonferroni corrections, indicating that all populations were substantially different from each other. Whangarei and Paengaroa were the most similar populations, while Te Kanuka and the Catlins were the most genetically distinct (Table 2.4).

Table 2.3 AMOVA analysis partitioning observed ISSR genetic variation between and within populations of *P. obcordatum*.

| Source              | df  | SS       | MS      | Est. var. | %    |
|---------------------|-----|----------|---------|-----------|------|
| <b>Between Pops</b> | 9   | 1283.798 | 142.644 | 10.431    | 44%  |
| <b>Within Pops</b>  | 118 | 1597.499 | 13.538  | 13.538    | 56%  |
| <b>Total</b>        | 127 | 2881.297 |         | 23.969    | 100% |

Table 2.4 Pairwise  $\Phi_{ST}$  comparisons between all sampled populations of *P. obcordatum*. These values were calculated in GenAlEx. All p-values were <0.005.

|                   | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     |
|-------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 Back Valley     |       |       |       |       |       |       |       |       |       |
| 2 The Catlins     | 0.504 |       |       |       |       |       |       |       |       |
| 3 Banks Peninsula | 0.319 | 0.467 |       |       |       |       |       |       |       |
| 4 Owen Valley     | 0.411 | 0.536 | 0.436 |       |       |       |       |       |       |
| 5 Te Kopi         | 0.466 | 0.592 | 0.558 | 0.384 |       |       |       |       |       |
| 6 Ahi Paku        | 0.467 | 0.548 | 0.517 | 0.419 | 0.301 |       |       |       |       |
| 7 Te Kanuka       | 0.512 | 0.639 | 0.623 | 0.529 | 0.531 | 0.308 |       |       |       |
| 8 Paengaroa       | 0.307 | 0.473 | 0.393 | 0.412 | 0.380 | 0.420 | 0.472 |       |       |
| 9 Whangarei       | 0.306 | 0.491 | 0.397 | 0.417 | 0.385 | 0.420 | 0.452 | 0.168 |       |
| 10 Kaitaia        | 0.493 | 0.584 | 0.543 | 0.548 | 0.539 | 0.553 | 0.553 | 0.381 | 0.323 |

The Mantel test found no significant positive correlation between genetic distance and geographic distance between all ten populations of *P. obcordatum* ( $R^2 = 0.0536$   $p = 0.129$ ).

## Discussion

The ISSR markers used in this study proved informative for measuring genetic diversity at both the species and population level. The ISSR markers produced 158 fragments that were reliably amplified and reproducible (Table 2.1; Figure 2.2).

Determining the number of private alleles is a useful process when studying the genetic uniqueness of a species. Private alleles are those that are unique to a single population among a wider collection of populations. Thus private alleles are useful for identifying a population as being genetically unique or divergent in comparison to other populations (Szpiech & Rosenberg 2011). A large number of private alleles may indicate an older divergence, which could be from

a period of long isolation (Petit et al. 1998; Bonin et al. 2007). Isolated populations are expected to have more private alleles because their reproductive isolation and lack of gene flow leads them to genetically diverge from other populations (Bonin et al. 2007). In this study, private alleles were found in Kaitaia (1), Paengaroa (1), Owen Valley (3) and Banks Peninsula (4). *Pittosporum obcordatum* had a low number of private alleles compared to populations of *P. cornifolium*. In the Clarkson et al. (2012) study on *P. cornifolium*, the offshore population on the Poor Knights Island was found to have 18 private bands, indicating a long period of geographic and genetic isolation. This may explain the relative abundance of private alleles in the Banks Peninsula population, as Banks Peninsula was previously an island separated from the main South Island (Bradshaw & Soons 2008; Wilson 2008; Wilson 2013). Likewise, Owen Valley is the only population of *P. obcordatum* west of the Main Divide, a mountain range that geographically isolates this population from others.

### Genetic diversity within populations

Expected heterozygosity ( $H_e$ ) showed that most populations of *P. obcordatum* have low to moderate amounts of population-level genetic diversity, with  $H_e$  ranging broadly from 0.083 to 0.201 (Table 2.1).

At the species level, *P. obcordatum* had a high amount of genetic diversity, demonstrated by the high level of polymorphism (P 94%; Table 2.2). When compared to other similar species (Table 2.5), this level was among the highest from the range, which had P ranging from 52.70% to 98%. Similar levels of polymorphism have been found in other *Pittosporum* species: *P. cornifolium* had a P of 90% (Clarkson et al. 2012) and *P. undulatum* had a P of 98% (Mendes et al. 2011).

Regionally, there was greater average diversity in the South Island populations (mean values of  $H_e$  0.167 and P 45.75%) compared to the North Island populations (mean values of  $H_e$  0.137 and P 40.02%). Average  $H_e$ , P and private alleles were all greater in the South Island populations. This is reminiscent of studies of *P. ferox*, where populations from the region between Banks Peninsula and Taieri/Waipori had the greatest genetic diversity in New Zealand. This was attributed to *P. ferox* being able to survive the Last Glacial Maximum at sites along the eastern coasts of the South Island (Shepherd & Perrie 2011).

At the population level, two of the populations sampled, Te Kopi ( $H_e$  0.086 and P 22.78%) and Te Kanuka ( $H_e$  0.083<sup>i</sup> and P 22.78%), had substantially less genetic diversity than the other eight populations. This could be attributed to genetic drift, including both founder effects and

genetic bottlenecks, as well as inbreeding. The Te Kopi site has been highly modified by exotic plantation and logging operations and as such now only consists of seven individuals (Chapter 1; Beadel et al. 2000). Loss of individuals from these sites has likely contributed to the lack of genetic diversity in these two populations.

Kaitaia ( $H_e$  0.142 and  $P$  37.34%) and the Catlins ( $H_e$  0.151 and  $P$  35.44%) were both small populations with moderate amounts of genetic diversity. The Catlins population had higher heterozygosity but the Kaitaia population had higher polymorphism. These populations despite being small, may have retained genetic diversity because the effects of genetic drift were not affecting them yet and they were recently larger. Back Valley ( $H_e$  0.157 and  $P$  46.3%) had relatively low genetic diversity compared to the other large population from Whangarei ( $H_e$  0.201 and  $P$  62.88%). It had been expected that Back Valley would have higher genetic diversity, as it was the least modified population due to the area not being cleared for human land use (Rance 2007). Instead, Back Valley was only the fourth most genetically diverse population. However, it is possible that the sample was not truly representative of the population because the range of individuals selected was too narrow due to time and identification constraints. Both the Back Valley and Whangarei populations had approximately 700 individuals, whereas Banks Peninsula had an  $H_e$  of 0.192 and a  $P$  of 61.39% with only 65 individuals.

In this present study, there were contrasts between population size and number of private alleles and genetic diversity. Whangarei had high genetic diversity but no private bands and is estimated to be the largest extant population (Table 2.1). Large populations tend to have higher genetic diversity because the effects of genetic drift are diluted and chances of non-random mating are reduced (Ellstrand & Elam 1993). There were no private alleles detected in Whangarei and this could be a consequence of previous genetic connectivity between this population and other northern populations. The population of Whangarei is approximately 100 km away from Kaitaia. There is also genetic evidence of past connectivity between Whangarei, Kaitaia and Paengaroa. Individuals from these populations were assigned to the same genetic cluster by STRUCTURE, for  $K=5$  (Figure 2.5).

Banks Peninsula is a small population that has relatively high genetic diversity, but in contrast to Whangarei has a high number of private alleles (4 private alleles and predominantly assigned to its own cluster; Figure 2.5). This high number of private alleles could be maintained because of geographic isolation and lack of connectivity with any other currently known populations over a long time span. Both geographic isolation and temporal isolation can contribute to a greater number of private bands due to mutation, random genetic drift, and local adaptation to different habitats (Baskauf et al. 2014). Banks Peninsula at different times has been isolated from the

mainland and *Myrsine divaricata*, a species that occurs with *P. obcordatum* at many locations, has been speculated to have survived there during glacial periods (e.g. Otira Glaciation, ~18,000 years ago) (Wilson 2008; Wilson 2013).

Small populations have greater extinction risk because of the loss of genetic diversity due to random genetic drift and increased inbreeding (Honnay & Jacquemyn 2007). In this study, a correlation was found between genetic diversity and population size. The smaller populations of *P. obcordatum* contained less genetic diversity than larger populations (Table 2.1). Because the sampling size varied between populations (Table 2.1), it can be hard to determine if patterns of genetic diversity are representative. For example, the low genetic diversity found in the population from Te Kanuka could be an artefact of small sampling size. However, when this was accounted for by a rarefaction technique (Leburg 2002, Coart et al. 2005), the result was significant, suggesting that small populations did contain less genetic diversity than larger populations.

This is a common pattern found in population genetic studies of plants with fragmented distributions, and the effect is more pronounced in outcrossing species than self-compatible species (Ellstrand & Elam 1993; Leimu et al. 2006; Honnay & Jacquemyn 2007). *Pittosporum obcordatum* is dioecious and hence predominantly outcrossing. It is assumed to be entomophilous, and this could be affecting genetic diversity, as small populations may be isolated from insect pollinators (Baskauf et al. 2014). Additionally, small populations may have skewed sex ratios with few males able to contribute to pollen, or few females to contribute ovules. This can lead to low fruit and seed set, and related offspring in a population (Ellstrand & Elam 1993; Hilfiker et al. 2004).

Compared to ISSR studies of other species that are outcrossing and rare, *P. obcordatum* had a lower mean  $H_e$  (0.149), than the compiled mean (0.179) but the means for PPL were approximately the same (44.22 vs 44.28%) (Table 2.5). Levels of genetic diversity in *P. obcordatum* were very similar to those found in the study by Clarkson et al. (2012) on *P. cornifolium*, with P ranging from 16.8 to 60.9%.

Table 2.5 Taxa used to compare genetic diversity within populations and genetic structure between populations. All studies used ISSR data and had outcrossing mating systems. *Pittosporum cornifolium* is an invasive species included, denoted by \*. All other taxa were rare. Included is information about percentage of polymorphic loci (P), expected heterozygosity ( $H_e$ ), and species estimates of  $\Phi_{ST}$ . The *P. obcordatum* results of this study were not included when calculating the means. Refer to Chapter 1 for census sizes citations.

| Family         | Species                        | Habit          | Mean P | Total P | $H_e$  | $\Phi_{ST}$ | Reference             |
|----------------|--------------------------------|----------------|--------|---------|--------|-------------|-----------------------|
| Burseraceae    | <i>Boswellia sacra</i>         | Tree           | 44.2   | 97.9    | 0.136  | 0.39        | Coppi et al. 2010     |
| Calycanthaceae | <i>Chimonanthus grammatus</i>  | Shrub          | 35.7   | 52.7    | 0.119  | 0.25        | Jiang et al. 2012     |
| Ericaceae      | <i>Rhododendron aureum</i>     | Shrub          | 54.78  | 95.16   |        | 0.38        | Liu et al. 2012       |
| Lamiaceae      | <i>Mentha cervina</i>          | Perennial herb | 44.4   | 98.3    |        | 0.49        | Rodrigues et al. 2013 |
| Lauraceae      | <i>Litsea szemaois</i>         | Tree           | 38     | 87.01   | 0.186  | 0.27        | Ci et al. 2008        |
| Pittosporaceae | <i>Pittosporum cornifolium</i> | Epiphyte       |        | 90.7    |        | 0.45        | Clarkson 2011         |
|                | <i>P. obcordatum</i>           | Shrub          | 44.28  | 94      | 0.149  | 0.44        | This study            |
|                | <i>P. undulatum</i> *          | Tree           |        | 98      |        |             | Mendes et al. 2011    |
| Polygonaceae   | <i>Rheum officinale</i>        | Perennial herb | 29.14  | 95.24   | 0.1008 | 0.74        | Wang et al. 2012      |
| Ranunculaceae  | <i>Ranunculus cabrerensis</i>  | Perennial herb | 38.59  | 81.38   |        | 0.31        | Cires et al. 2013     |
| Rosaceae       | <i>Hagenia abyssinica</i>      | Tree           | 73     | 81      |        | 0.25        | Feyissa et al. 2007   |
| Rutaceae       | <i>Citrus hongheensis</i>      | Tree           | 36.5   | 95.1    | 0.352  | 0.64        | Yang et al. 2010      |
| Means          |                                |                | 43.49  |         | 0.179  | 0.42        |                       |

A meta-analysis by Nybom (2004) using RAPD markers found a mean  $H_e$  of 0.27 for outcrossing species and a mean of 0.25 for long-lived perennials, indicating the  $H_e$  of *P. obcordatum* is relatively low. *Pittosporum obcordatum* still has comparatively higher genetic diversity than *Chimonanthus grammatus*, an outcrossing endangered shrub from China, with a low mean genetic diversity ( $H_e$  0.119), and population level  $H_e$  ranging from 0.06-0.154 (Jiang et al. 2012).

Despite most sites of *P. obcordatum* that were sampled having populations of <100 individuals, *P. obcordatum* still showed relatively moderate levels of genetic diversity. This could be attributed to a predominantly outcrossing mating system which limits self-pollination and the resulting increase in homozygosity. In addition, the estimated 120-year lifespan of *P.*



*obcordatum* individuals suggests that there could be a long generational lag time maintaining genetic diversity, as some of the individuals may have been alive prior to habitat fragmentation (Clarkson & Clarkson 1994).

### **Genetic structure between populations**

The results of the AMOVA, pairwise  $\Phi_{ST}$  distances, and STRUCTURE analyses indicate that populations of *P. obcordatum* are genetically distinct from each other and suggest a strong relationship between genetic structure and geography. There is little evidence of gene flow between populations, which may be explained by geographic distance as most populations are 100 – 1,300 km away from each other (Appendix I).

Although the  $\Delta K$  STRUCTURE parameter indicated  $K=9$  as the most probable number of genetic clusters, this did not make biological sense and did not capture the major structure of the data (Pritchard et al. 2000). However, both the  $\Delta K$  and the  $L(K)$  showed support for  $K=5$ , so for this study five genetic clusters are more biologically meaningful. For  $K=9$ , individuals were not always assigned with populations of origin. However, generally under both  $K=9$  and  $K=5$ , genetic clusters corresponded with geographical patterns, and population similarities corresponded with relative proximity to one another. The pairwise  $\Phi_{ST}$  values were high and all were significant, further indicating strong structure and lack of gene flow. The results of the cluster analyses ( $K=5$ ,  $K=9$ ) and the pairwise  $\Phi_{ST}$  values supported the Back Valley and Owen Valley populations as being genetically divergent from all other populations. Both populations are geographically isolated from other populations, Back Valley by distance and latitude, while Owen Valley is the only South Island population west of the Main Divide. The isolation of Owen Valley by mountain ranges may be significant in terms of its genetic isolation, because a major mountain range is more of a barrier to gene flow than simple distance. The Owen Valley population may be showing localised adaptation to very specific environmental conditions, or its divergence could be due to genetic drift over time and not necessarily because of selection (Ellstrand & Elam 1993; Frankham et al. 2009).

Alternatively, the north-west Nelson region has been hypothesised as a glacial refugium by Gardner et al. (2004) as part of their study on *Metrosideros excelsa*. It has been suggested that many lowland forest trees may have survived glacial periods in microsites, with theoretical refugia for lowland species being located in the northern and southern parts of the South Island (Gardner et al. 2004; Shepherd & Perrie 2011). The Owen Valley may therefore have been a glacial refugium, the genetic divergence of its *P. obcordatum* population reflecting long-term

isolation. If this was the case, it would be expected that surrounding populations would show a subset of its genetic diversity (Shephard et al. 2007). Unfortunately, no surrounding populations are known, so this hypothesis cannot currently be tested.

The results of the AMOVA analysis indicate that genetic variation was proportioned as 44% between populations while 56% of the variation was within populations (Table 2.3). This result was similar to *P. cornifolium* (59.81% within), although its populations were geographically closer together (Clarkson et al. 2012). This is an expected result, as outcrossing long-lived perennials tend to contain most of their genetic variation within populations (Nyborg 2004). Genetic differentiation between populations was substantial, as the overall  $\Phi_{ST}$  of 0.44 indicates there is either none or limited gene flow between populations (Wright 1965). The  $\Phi'_{ST}$  value of 0.525 shows that in the case of *P. obcordatum*, within-population variation is not depressing overall  $\Phi_{ST}$  values. This  $\Phi_{ST}$  value is higher than the average value of the compilation of rare outcrossing perennials (0.419) (Table 2.4) and also higher than values of dominant markers reported by Nyborg (2004) for long lived perennials (0.25) and for outcrossing plants (0.27).

Disjunct populations that occur over a wide range tend to have minimal gene flow, and geographic distance between populations is a major factor in this as it limits the amount of gene flow by pollen and seeds (Pfeifer & Jetschke 2006). The high  $\Phi_{ST}$  values of these populations can be attributed to dispersal and pollination syndromes as well as decreased population size and potential lost connecting populations. Dispersal of *P. obcordatum* seeds is unknown but that of the *Pittosporum* genus is assumed to be by bird or by occasional gravity (Burrows 1994; Clarkson & Clarkson 1994; Gemmil et al. 2002). The high  $\Phi_{ST}$  value is very similar to the average value for gravity dispersed plants of 0.45, but in contrast, much higher than dispersal by ingestion (0.27) (Nyborg 2004). The populations of *P. obcordatum* are substantially isolated from each other, and since the arrival of mammals on the mainland, bird dispersers have dramatically reduced in number, so this high  $\Phi_{ST}$  number could be a consequence of dispersal failure (Kelly et al. 2004).

In the North Island, the three populations of the Eastern Wairarapa were grouped together by cluster analyses and the pairwise  $\Phi_{ST}$  values, an unsurprising result given their close geographical distance. The population of Paengaroa proved more surprising, as it is geographically closer to the eastern Wairarapa than the Northland (Whangarei and Kaitia) populations with which it grouped in cluster analyses and pairwise  $\Phi_{ST}$  values (<200 km separates Paengaroa and the eastern Wairarapa vs 470 km between Paengaroa and Whangarei; Appendix I; Chapter 1). Shepherd and Perrie (2011) found a similar cluster when studying *P. ferox*: the population situated at Moawhango (~24 km from Paengaroa) clustered

with Northland and Auckland populations rather than Rimutaka and the northern South Island populations.

The genetic data in this study shows no support for var. *kaitaiaensis*. Whangarei and Kaitaia, although still significantly different from each other (Table 2.4), showed evidence of previous gene flow, as they were clustered together in the STRUCTURE analyses.

Analysis of all sampled populations of *P. obcordatum* showed no pattern of isolation by distance. This is in accordance with the pairwise  $\Phi_{ST}$ , which showed that even populations that were geographically closer were substantially differentiated from each other (Table 2.4). Vicariance due to habitat loss and life history traits of *P. obcordatum* such as dispersal and pollination syndrome might also be contributing to the genetic differentiation between the populations.

#### Comparison to other New Zealand tree species

Some plants, such as *Olearia gardneri* (Asteraceae), have low genetic diversity and low differentiation, indicating that in the past, populations had been highly connected. Other disjunct plant species such as *V. speciosa* have lower genetic diversity (attributed to small population size) and marked genetic differentiation between populations, suggesting no gene flow occurred prior to habitat loss (Armstrong & de Lange 2005). The results from this study of *P. obcordatum* indicate that it has low to moderate genetic diversity and substantial genetic differentiation, suggesting that it has little gene flow between current known populations.

Population structure in New Zealand's disjunct plant species has also been previously associated with latitude. A study of *Cordyline australis* (Beever et al. 2013) split populations into groupings North or South of Lake Taupo, with genetic diversity progressively decreasing southwards. The study of *V. speciosa* also found less genetic diversity in southern populations (Armstrong & de Lange 2005). Not all population structures gradate to the south, however. For instance, *P. ferox* has notably greater genetic diversity in southern populations (Shepherd & Perrie 2011). The present study found no evidence of southern populations of *P. obcordatum* being more genetically depauperate than northern populations. On the contrary, genetic diversity appeared to be higher within the southern populations.

#### **Conservation implications and conclusions**

Knowledge of genetic diversity within and between populations provides effective information for conservation management plans for New Zealand's endangered species. Conservation of

genetic diversity safeguards the evolutionary potential of species. The genetic clusters and the number of private alleles identified in this study suggest populations in the South Island contain unique genetic variation which needs to be conserved. These populations should be monitored to minimise population loss, and be maintained as separate management units. Owen Valley and Banks Peninsula in particular are populations requiring active monitoring, as there are unique alleles and significant diversity in both populations. As unique populations, all restoration should occur only from seeds collected within the area, and seeds should be sourced from as many different individuals as possible (McKay et al. 2005; Malaval et al. 2010). Owen Valley is of significant concern because of its small population size, which should not be allowed to decrease any further. Conservation should entail growing seeds from as many individuals as possible from Owen Valley.

The Catlins population is at risk due to its small population size, fragmentation and isolation of individual trees, and there are additional problems in this area due to encroaching invasive species (J. Barkla, pers.comm.). Monitoring of the Catlins population should involve determining the true extent of the Catlins population and the study of the unsampled Southland populations as potential seed sources for any restoration.

The Back Valley population has ecological value as a continuous relatively intact habitat, that has never been cleared for agriculture (Rance 2007). Its genetic diversity was moderate, and currently could be monitored for population size. Both the Back Valley and Whangarei populations require no active management of genetic diversity at this time.

In the North Island, the most significant population in terms of genetic diversity is Whangarei, followed by Paengaroa. While the Whangarei population is of little conservation concern at this time, Paengaroa would benefit from the same active restoration as Owen Valley, by increasing population size with plants grown from Paengaroa seeds. Te Kanuka and Te Kopi are currently at high risk because of their low genetic diversity and small population sizes. However, these two populations, along with Ahi Paku, are clustered together and genetically similar enough that it may be prudent to translocate seedlings between these three populations. Kaitaia has low genetic diversity, a small population size and only one known female plant. Active monitoring and restoration is required to restore this population. Based on genetic distances and clustering, it would be appropriate to source seed from Whangarei populations.

For the short- and long-term persistence of *P. obcordatum* populations in the wild, it is important to ensure populations do not get any smaller. Frankham et al. (2014) recommend a population size of at least 100 individuals to minimise the effects of inbreeding. As outlined earlier, most

populations of *P. obcordatum* contain <100 individuals, and because his study found that smaller populations had less genetic diversity than larger populations it is imperative that some form of restoration is implemented.

The overall genetic diversity of the sampled populations of *P. obcordatum* is moderate in comparison to plant species with similar characteristics. Smaller populations of *P. obcordatum* had less genetic diversity than larger populations, but no latitudinal cline of genetic diversity was found. The smaller populations of *P. obcordatum* are in danger of inbreeding if the populations are not actively managed and continue to decrease in population size. Populations of *P. obcordatum* were substantially differentiated from each other throughout its range. Three of the populations stood out as being genetically unique, and for this reason, they should be conserved as separate management units.

This study was a preliminary study to investigate the genetic structure of *P. obcordatum*. As it did not sample all known populations of *P. obcordatum*, the Hawke's Bay and Southland populations should be sampled in any future study of the conservation genetics of this species. It is possible that not sampling the Hawke's Bay and Southland population influenced the genetic structure found in this study. However, as sampling occurred in areas near the unsampled populations (The Catlins near Southland and in Hawkes Bay) no significant influence is expected from these populations.

This study used ISSR markers as they are inexpensive and provide reproducible results. It is recommended that any future studies reassess the populations by using co-dominant markers such as microsatellites, as this would provide a more accurate measure of genetic diversity and allow heterozygotes to be distinguished from dominant allele homozygotes. Phylogeographic analyses, such as those on Zealandic *Pittosporum* species (Gemmill et al., in Prep), will be of benefit to understanding the evolutionary and biogeographic history these species. These can be complimented with Bayesian divergence analysis if appropriate fossils can be located, to give a better understanding of the time frames associated with the diversification of New Zealand *Pittosporum* species.

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## **Chapter 3: How can *Pittosporum obcordatum* grow in an atypical habitat on Banks Peninsula? Drought tolerance and soil conditions in a hillslope population of *P. obcordatum* on Banks Peninsula**

### **Introduction**

*Pittosporum obcordatum* (Pittosporaceae) is an endangered lowland plant species that appears to have a very specialised habitat preference, as outlined in Chapter 1. Clarkson & Clarkson (1994) emphasised that the habitat of *P. obcordatum* is lowland alluvial forest, and that plants were sometimes found on colluvial toeslopes but not on hillslopes. The locations where *P. obcordatum* grow are often subject to flooding and frosts in winter, and drought in summer (Clarkson & Clarkson 1994). Another unusual feature of the typical habitat is that it has a high proportion of small leaved and divaricating shrubs and an absence of broadleaved species (Clarkson 1991; Clarkson & Clarkson 1994). Rogers (1996) attributed the rarity of *P. obcordatum* in the North Island to it being adapted to a habitat with relatively “extreme” seasonal edaphic and climate conditions.

### *Pittosporum obcordatum* on Banks Peninsula

My aim in this chapter is to test some aspects of the habitat preferences of *P. obcordatum* on Banks Peninsula, on the east coast of the South Island. Vegetation cover on Banks Peninsula prior to human settlement was mainly podocarp-hardwood forest, with prominent lowland trees including *Prumnopitys taxifolia* (matai), *Kunzea ericoides*, *Melicytus ramiflorus* and kahikatea (Wilson 2013). Banks Peninsula was settled by Maori after 1280 AD and by about 1800 AD some 35 % of the old growth forest had been cleared and replaced largely by grassland. After European settlement on Banks Peninsula in the 1840s, deforestation continued and only 1% of Banks Peninsula remains in old growth forest (Ewers et al. 2006; Wilson 2008; Wood & Pawson 2008).

*Pittosporum obcordatum* was first collected and described by E.F.L. Raoul, in the 1840s on Banks Peninsula (Raoul 1844), but not recorded again from that area until recently. In 2012, this species was rediscovered on Banks Peninsula, where it had been presumed locally extinct (Wilson 1992; Clarkson & Clarkson 1994; Wilson 2013; Hutchison 2014). Since 2012, 65 plants have been found on Banks Peninsula in a valley near Okains Bay amongst secondary growth, mixed shrubland. The plants occur on a south-east facing slope of land and the land is grazed

by sheep and cattle (Hutchinson 2014). *Carex inopinata* and *Olearia fimbriata* are other 'Nationally Vulnerable' species occurring at this site (de Lange et al. 2013).

The parent material of the soil on Banks Peninsula is colluvium, which is a mixture of loess and igneous bedrock (Webb 2008). The soil on the peninsula is variable, but overall it is well-drained and of moderate fertility (Wilson 2008). According to Griffiths (1973), there are two main types of loess on Banks Peninsula: Birdlings Flat loess and Barrys Bay loess. Griffith categorised the parent soil that occurs in the *P. obcordatum* habitat in Okains Bay as Barrys Bay loess, specifically Pawson soil (Fine, sandy loam silt; Griffiths 1973; Webb 2008). Pawson soil is a mixture of yellow-grey earth and yellow-brown earth. It tends to be compacted, poorly drained and prone to gleying (Wilson 1992; Wilson 2013).

There are examples of New Zealand plant species that are restricted to very specific environmental conditions. For example, the New Zealand *Sophora* genus include both habitat generalists and specialists. The three widespread *Sophora* species: *S. microphylla*, *S. tetraptera* and *S. chathamica* can compete well on fertile soils, while five other species *S. longicarinata*, *S. fulvida*, *S. godleyi*, *S. prostrata* and *S. molloyi* are more habitat specific but specialised to habitats that are harsher (e.g. being rocky, infertile, calcareous, dry, and/or windy) (Heenan 1998; Thomas and Spurway 2002).

However, there have been examples of New Zealand plant species, such as *Carmichaelia (Notospartium) glabrecens*, that are not as habitat specialised or as rare as once thought, and this could be the case with *P. obcordatum* (Heenan 1996). It is possible that *P. obcordatum* was formerly widespread in lowland forests, and its current scarcity is in fact the result of habitat loss. Environmental factors that affect a species' distribution, especially the distribution of an endangered habitat specialist, need to be considered so that we know where to look for them and also to cope with any future changes to their habitat (Preston et al. 2008). The habitat range for many specialists may correlate strongly with an ability to tolerate extremes of temperature, soil composition or moisture (Preston et al. 2008). These could be further affected by topography such as hillslope gradient or aspect (Bennie et al. 2006).

Ecotypes can evolve when populations of a species adapt to local environmental conditions. For example, populations of *Weinmannia racemosa* (kamahi) from montane habitats are more cold-hardy than those from lowland habitats (Sakai and Wardle 1978), and *Fagus sylvatica* seedlings from wetter habitats tend to be more drought-sensitive than those from drier habitats (Peuke et al. 2002).

Soil conditions are a major factor influencing the distribution of plant species. Some plants seem to be specialised to soil types. For instance, in New Zealand there are plants known as calcicoles, which appear to be restricted to limestone based soils and outcrops. They include: *Veronica brevifolia* (*Hebe brevifolia*), *Carex ophiolithica* and *Pittosporum ellipticum* subsp. *serpentinum* (de Lange 1997; de Lange & Heenan 1997; de Lange 1998). Molloy (1994) uses 'basicole' for a broader group of plants that is restricted to all basic soils, not just those derived from limestone.

#### Justification and aims of ecological experiments

Until the Banks Peninsula population was discovered, populations of *P. obcordatum* were known only from fertile alluvial lowland forest habitats and floodplains, typically in swampy areas (Clarkson & Clarkson 1994). However, the Banks Peninsula population grows on an ocean-facing hillslope, so either the sites are more ecologically/biologically similar than they appear, or *P. obcordatum* has a wider ecological tolerance than previously thought. Determining which of those alternatives is correct is important for guiding the conservation and management of the new population, to refine search strategies involved in finding more populations, and to assist in understanding the specific requirements of *P. obcordatum* to persist in the long term (e.g. some species need regular disturbance to open up space and light to grow; Begon et al. 2006). Here I aim to test two hypotheses about the unexpected habitat of *P. obcordatum* on Banks Peninsula. They are:

(1) *Pittosporum obcordatum* is able to grow on a hillslope on Banks Peninsula because that population has adapted to drier conditions than the populations on alluvial flats elsewhere in New Zealand.

(2) *Pittosporum obcordatum* grows on a hill slope on Banks Peninsula because the soil there is as fertile as the soil of alluvial flats.



## Materials & Methods

### Drought experiment

The aim of this experiment was to see if the Banks Peninsula population of *P. obcordatum* is able to grow on a hillside because it is more drought tolerant than the populations growing on alluvial flats. Experiments like these have been used to test for local adaptation in other plant species e.g *F. sylvatica* (Rose et al. 2009).

*Pittosporum obcordatum* seeds were collected from all locations where they were present. Fewer seeds were collected at some populations because no female plants were found or the height of the branches made seed collection difficult. Moore et al. (1994) suggested that *P. obcordatum* seeds most likely have a stratification requirement. Therefore, I only collected last season's seeds to ensure that they were on the plant for at least one winter in order to meet stratification requirements.

Seeds were collected from Owen Valley, Back Valley and The Catlins in December 2013 and from Ahipaku, Whangarei, Kaitaia and Banks Peninsula in February 2014. No seeds could be found on any of the Paengaroa and Te Kanuka plants. The Te Kopi site was not included in field work in the North Island (See Chapter 1).

In April 2014, seeds from these seven sites were planted in trays filled with potting mix separated by source population. Potting mix composition was 80% "Hort Bark" and 20% Blood and Bone with fertiliser. Trays were set in a larger tray filled with water (for slow absorption) in a growth cabinet for two months. The growth cabinet environment was set at 16 hours of light at 21°C and 8 hours of darkness at 10°C. The first seedlings started emerging in May 2014.

In June 2014, all seedlings (1-6 cm tall) were planted in pots that were 65 mm in height and 70 mm in diameter containing 60 g of sieved potting mix. Seedlings were then moved into the University of Canterbury glasshouses. Seedlings were watered for three minutes every morning by an automatic sprinkler system.

### Trial drying period

The low number of seedlings that germinated from sites apart from Banks Peninsula (see Results) indicated the need for a pilot study to determine an appropriate duration of the drought treatment. To establish a base measurement of drought survivorship, Banks Peninsula

seedlings were used in this trial. I randomly selected fifty Banks Peninsula *P. obcordatum* seedlings and ceased to water them on October 9<sup>th</sup> 2014. Then at different stages (Table 3.1), groups of ten seedlings were rehydrated and their survival was assessed by determining stem wilt, persistence of green leaves, or renewed growth.

Table 3.1 Survival of *P. obcordatum* seedlings from Banks Peninsula in ten day trial drying period. Plants were labelled P1-50.

| Plants  | Days in drought | % survival |
|---------|-----------------|------------|
| P1-P10  | 5               | 100        |
| P11-P20 | 7               | 100        |
| P21-P30 | 10              | 80         |
| P31-P40 | 14              | 50         |
| P41-P50 | 18              | 10         |

Plants were all watered for three minutes every day in the University of Canterbury glasshouses for six weeks to see how well they recovered. Half of the seedlings that were exposed to a fourteen day drought died (Table 3.1). I decided that the main drought treatment would go for ten days (instead of fourteen). This was because of the difference in mean air temperature between October 2014 when the trial was run and January 2015, when the main experiment was run (17.5 °C and 23.5 °C, <http://Cliflo.niwa.co.nz>). The increase in mean air temperature would cause plants to dry out faster than during this trial.

### The drought experiment

In order to investigate the potential for seedlings to resist drought, I subjected 81 seedlings to ten drought days. The experiment was conducted in January 2015 and used two plant populations (Banks Peninsula and Back Valley). This was because of the poor germination of seeds from the other populations. All seedlings were approximately 7-8 months old and ranged in height from 7 cm to 31 cm. Because there were only 22 seedlings from Back Valley, only two treatments were used in the final experiment.

In each treatment (drought and control), there were seventy individuals from Banks Peninsula and eleven individuals from Back Valley. Plants were randomly assigned an identification number and leaf numbers and plant heights were recorded. Leaves over 5 mm on the main

stem were recorded as a measure of plant size (leaf number). It was assumed that plants under drought stress would lose more leaves, as leaf senescence is an indicator of drought stress (Guo & Gan 2014).

At the start of the experiment all plants were hydrated by setting them in a tray full of water for thirty minutes. To minimise the effects of shade and position in the glasshouse, plants were randomly moved around within their treatment area once a week. Plants assigned to the control treatment were watered under the standard watering regime, while plants under the drought treatment remained unwatered for ten days. After ten days, all plants were rehydrated and returned to the usual watering regime.

After six weeks of recovery time, the number of seedlings that survived and leaf number were recorded. All leaves over 5 mm on the main stem were counted to gain a measure of leaf senescence. *Pittosporum obcordatum* seedlings were considered dead when leaves and stem turned orange and no regrowth occurred.

The effects of the drought treatment experiment were analysed in two different ways. First, by recording survival under the drought treatment compared to survival under the control treatment. Secondly, by recording leaf numbers before and after the two treatments.

To determine differences of survival between the two treatments and two populations, I used a binomial Generalised Linear Model (GLM) run in R version 3.1.1 (R core development team 2014). A  $\chi^2$  test was used to test significance instead of an F-test because the data distribution was binomial. I checked for over-dispersion in the data but there was none.

To test for the effect of treatment on leaf loss of surviving plants, leaf numbers on each seedling were recorded both before and after the two treatments. The ratio for each individual's leaf loss was calculated as final/initial. To see if the ratios were normally distributed a qqnorm plot was created and because they were right skewed I took the log of the ratio ( $\log_{10}(\text{final/initial})$ ). This made the data normal enough so then a gaussian GLM was used.

All statistical analyses were conducted using the statistical package 'R' (R core development team 2014).

### **Phytometer experiment**

A phytometer experiment was used to determine whether soil conditions on the Banks Peninsula

hillslope could support plant growth better than alluvial flat sites. Phytometers are an easy and inexpensive way to measure soil site differences by comparing the rate of increase of plant biomass (Dietrich et al. 2013). Radish (*Raphanus sativus*) seeds were chosen because they grow quickly, and have been used in phytometer studies previously (Köhler et al. 2001; Albrecht et al. 2007; Axmanová et al. 2011).

Between December 2013 and March 2014, soil was collected from three alluvial flat sites (Back Valley, Owen Valley and Kaitaia) and the hillslope site of Banks Peninsula. Six core samples were collected from four different areas in each site, giving 24 cores of soil from each of the four sites. A metal corer was used (9 cm deep, by 7 cm internal diameter) with hollow plastic cylinder inside to ensure the same volume of soil was collected with each core. There was a pin through the top of the tube to ensure the plastic piping stayed in place. Soil from each location was stored dry in plastic snap lock bags in a dark cupboard until ready to be used for the phytometer experiment.

In January 2015, the soil from all 24 cores from each location were sieved, homogenised and divided over six pots (70 mm deep and 80 mm in diameter), giving a total of six pots from each of the four locations (24 pots in total). Following the methods of Axmanová et al. (2011), nine radish seeds were placed in each pot in groups of three and left in the glasshouse to germinate. After two weeks growth, six of the seedlings were removed, leaving the largest three seedlings in each pot. Pots were watered for three minutes every day for six weeks. At the end of the phytometer experiment, all radishes were harvested, loose soil was shaken off and the three entire radish plants (roots and shoots) from each pot were put into a paper bag and dried in an oven at 60°C for 72 hours. They were then removed from the oven and weighed immediately.

To compare phytometer growth in soils from the four locations, I first created a qqnorm plot to see if the dry weights were normally distributed. Because the data were right skewed, I took logs of the dry weights. The productivity of soil from each location was compared using a one-way Anova. To determine if locations were significantly different from each other a post-hoc Tukey test was run in R (R core development team 2014).

## Results

### Drought experiment

#### Seed germination

Plants at the Banks Peninsula location had abundant seed which meant that more seed could be collected. The Banks Peninsula population also had the highest germination rate with 82% of sown seeds germinating. This meant the number of germinated seedlings for Banks Peninsula was larger. Back Valley was the other population used for this experiment because it had a larger number of seedlings than the other populations (40%). The Catlins population had a 35% germination rate; however there was only seven seedlings, which was too few to use in the drought experiment. All remaining populations showed little or no germination.

Table 3.2 Seed germination rate from eight populations of *P. obcordatum* sampled.

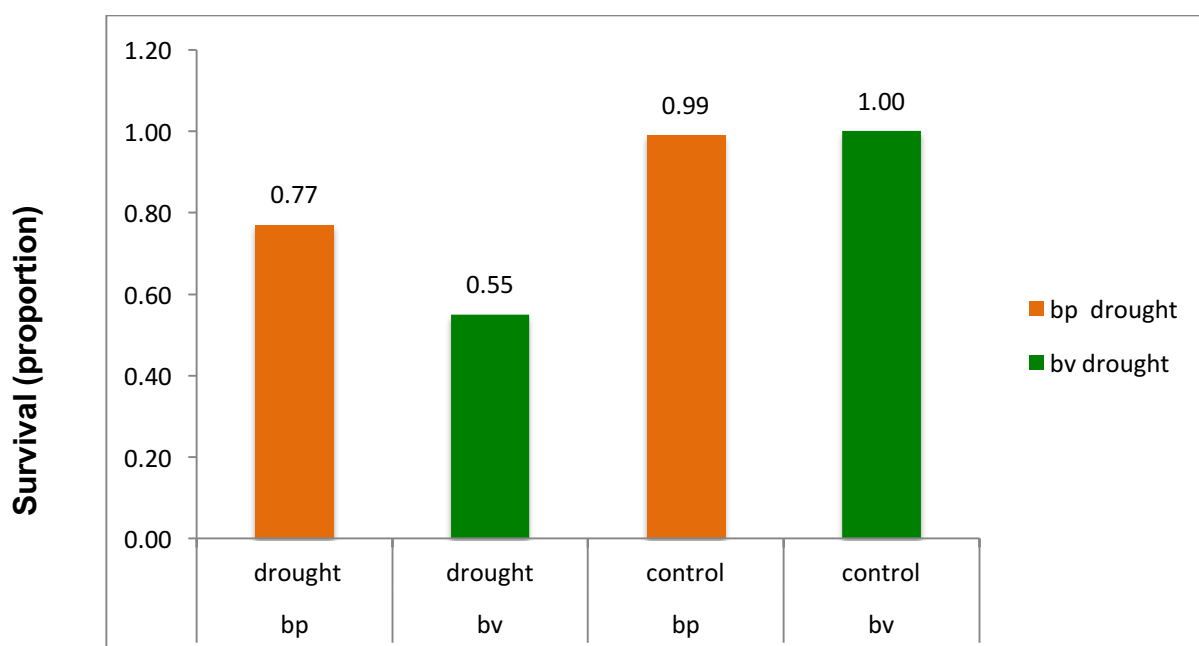
| Site            | Collected | Germinated | % Germinated |
|-----------------|-----------|------------|--------------|
| Banks Peninsula | 305       | 250        | 82           |
| Owen Valley     | 50        | 0          | 0            |
| The Catlins     | 20        | 7          | 35           |
| Back Valley     | 100       | 40         | 40           |
| Ahipaku         | 20        | 1          | 5            |
| Paengaroa       | 0         | 0          | 0            |
| Whangarei       | 80        | 0          | 0            |
| Kaitaia         | 20        | 0          | 0            |

#### Drought experiment

The first variable measured for the drought stress experiment was the survival of seedlings from Banks Peninsula and Back Valley. More seedlings died in the drought treatment compared to the control treatment for both populations (Figure 3.3). The effect of the drought treatment was

statistically significant ( $P < 0.0001$ , Table 3.3). In the drought treatment for the Back Valley population, 45% of the seedlings died and for Banks Peninsula, 22.8 % of the seedlings died. In the control treatment, none of the plants from Back Valley died, while 1.4% of the seedlings from Banks Peninsula died. The effects of population and the interaction of treatment and population were non-significant (Table 3.3). Hence, although a higher proportion of seedlings from Back Valley died in the drought treatment than from Banks Peninsula, the non-significant interaction means I cannot be sure this a real difference between the populations.

For surviving plants, leaf count varied significantly with treatment, with control plants having significantly higher ratios compared to treatment plants. The leaf count ratios showed that both populations increased in leaf number in the control treatment, whereas both populations decreased in leaf number in the drought treatment (Figure 3.4 leaf ratios  $>0$  and  $<0$  respectively, and Table 3.5  $>1$  and  $<1$  respectively). In the drought treatment, the median leaf ratio was lower for Back Valley plants, suggesting that survivors coped less well with drought than Banks Peninsula plants, but this was not significant. The GLM showed a highly significant effect of treatment, but no significant effect of population or, crucially, the population and treatment interaction (Table 3.4).



### Treatment and population

Figure 3.3 Proportional survival of two populations of *P. obcordatum* seedlings after a drought experiment. BP = Banks Peninsula BV = Back Valley

Table 3.3 GLM results testing survival of *P. obcordatum* seedlings against treatment and population. P values <0.05 are indicated with \*

| Variable             | Df | Deviance | Residual<br>Df | Residual<br>deviance | P value |
|----------------------|----|----------|----------------|----------------------|---------|
| NULL                 |    |          | 161            | 128.72               |         |
| treatment            | 1  | 25.23    | 160            | 103.49               | <0.001* |
| population           | 1  | 1.90     | 159            | 101.59               | 0.169   |
| treatment:population | 1  | 0.69     | 158            | 100.9                | 0.405   |

Note: df = degrees of freedom. P is significant at <0.05

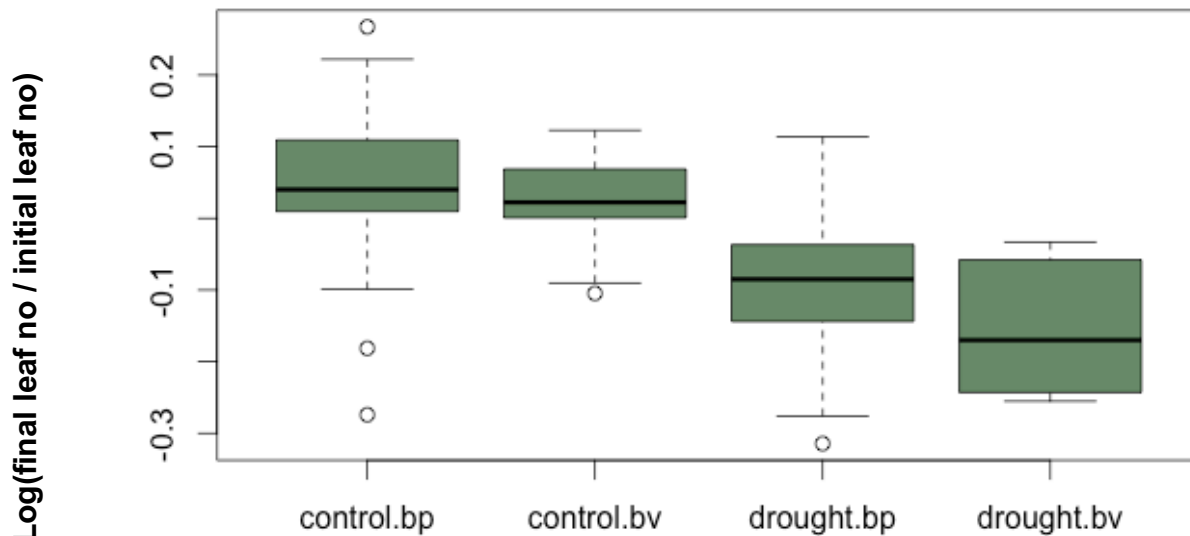
Table 3.4 GLM results of the relationship between the leaf count ratio of population, treatment and the interaction for *P. obcordatum* seedlings.

| Variable             | MS    | df  | F     | P value   |
|----------------------|-------|-----|-------|-----------|
| treatment            | 0.700 | 1   | 97.73 | <0.001*** |
| population           | 0.023 | 1   | 3.26  | 0.073     |
| treatment:population | 0.004 | 1   | 0.56  | 0.457     |
| Residuals            | 0.007 | 135 |       |           |

Note: MS= Mean Squares, df= degrees of freedom, F= F statistic. P is significant at <0.05

Table 3.5 Mean leaf count ratios (final leaf count/initial leaf count) for surviving *P. obcordatum* seedlings from the populations throughout the drought experiment.

| Population      | Treatment | Mean leaf count ratio | Standard error |
|-----------------|-----------|-----------------------|----------------|
| Banks Peninsula | Control   | 1.137                 | 0.03           |
| Back Valley     | Control   | 1.059                 | 0.05           |
| Banks Peninsula | Drought   | 0.820                 | 0.02           |
| Back Valley     | Drought   | 0.714                 | 0.06           |



### Treatment and Population

Figure 3.4 Box plot of the log of leaf count ratio comparing populations and treatment. bp = Banks Peninsula, bv = Back Valley. A log leaf ratio of 0 indicates no change in leaf number throughout the experiment

### Phytometer experiment

Of the four populations, Banks Peninsula soil produced the least phytometer biomass (Figure 3.5, Table 3.6). Of all the alluvial flats populations, Kaitaia performed the worst. Owen Valley and Back Valley alluvial flats both produced substantially more biomass, with means of more than twice the size of the other two populations (Table 3.6). The AMOVA high F value of 15.44 ( $P < 0.001$ ) means that mean plant growth varied significantly among the different soils (Table 3.7). The Tukey test showed that Banks Peninsula soil was significantly different from the three other alluvial flat sites (all P- values  $< 0.0001$ ). The other three sites were not significantly different from each other.



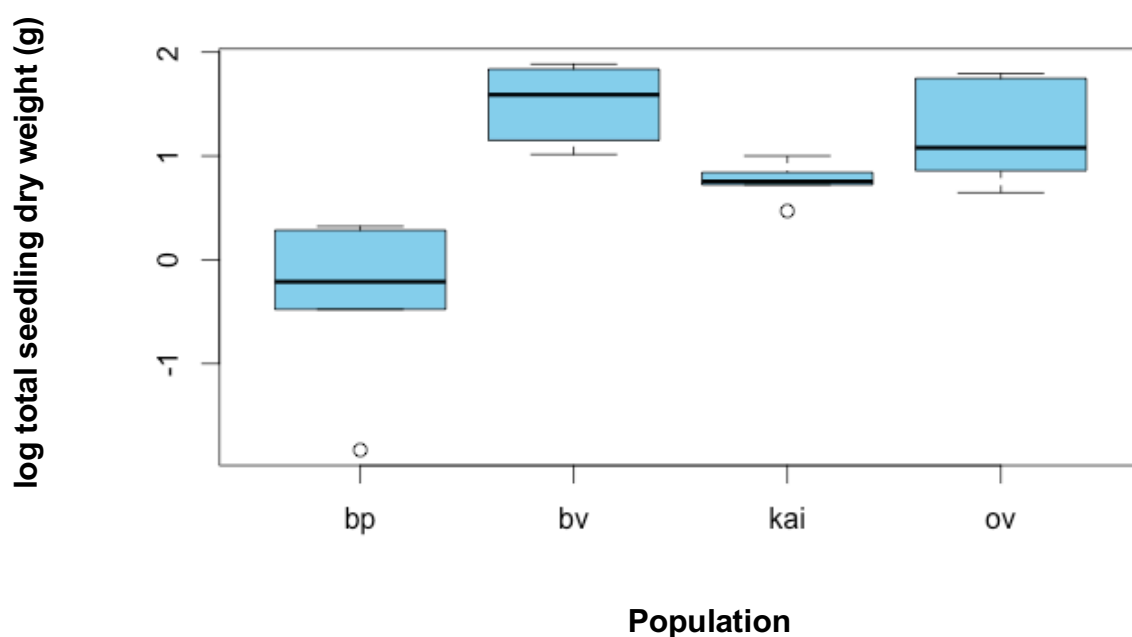


Figure 3.5 Total seedling dry mass (g) for radish plants grown for six weeks in soil from four locations. Bp = Banks Peninsula, bv = Back Valley, kai = Kaitaia, ov = Owen Valley .

Table 3.6 Comparative weights of radish seedling mass from the four locations. There were 3 plants in each of 6 pots per site.

| Location        | Mean (g) dry weight<br>per population | SEM  |
|-----------------|---------------------------------------|------|
| Kaitaia         | 2.16                                  | 0.15 |
| Back Valley     | 4.82                                  | 0.72 |
| Owen Valley     | 3.66                                  | 0.73 |
| Banks Peninsula | 0.85                                  | 0.19 |

Note: SEM= Standard error of the mean

Table 3.7 ANOVA results testing variation in log (seedling dry mass (g)) among soil from four sites.

|           | df | SS    | MS     | F     | P value |
|-----------|----|-------|--------|-------|---------|
| site      | 3  | 11.99 | 3.998  | 15.44 | <0.001* |
| residuals | 20 | 5.18  | 0.2598 |       |         |

Note: df= degrees of freedom, SS= Sum of Squares, MS= Mean Squares, F= F statistic and P is significant at <0.05

## Discussion

### Drought stress experiment

The drought stress experiment was conducted to determine whether greater drought stress tolerance might be present in the hillslope *P. obcordatum* population on Banks Peninsula. Plants growing on slopes may require greater drought resistance as hillslope habitats dry out faster than flats. Aspect and steepness of slope affect amount of radiation and moisture a hillslope receives and retains (Swanson et al. 1988). Additionally, plants growing on hillslopes in British chalklands have been shown to have greater drought tolerance (Bennie et al 2006). The Banks Peninsula location has a mean annual rainfall of less than 750 mm, while the Back Valley location has a mean annual rainfall of 3786 mm (Wilson 1992; Mark et al. 2001; Wilson 2013;). Survival of *P. obcordatum* trees on a hillslope on Banks Peninsula with comparatively low rainfall suggests a capacity to withstand drought.

Survival of seedlings from the Back Valley and Banks Peninsula populations was significantly affected by the drought stress treatment. No significant evidence was found that seedlings from the Banks Peninsula population were more drought tolerant than those growing on an alluvial flat at Back Valley, Fiordland. Since the interaction of treatment and population is non-significant, the Banks Peninsula population does not appear to be more drought tolerant than the Back Valley population. This indicates that the level of drought stress chosen to challenge the seedlings was appropriate, because a number of seedlings exposed to drought died, while some survived. The results of the drought tolerance experiment suggested a greater capacity to survive drought in Banks Peninsula seedlings, but this was not significant, perhaps because the seedling numbers from comparative populations were too low. At present, I can only conclude that I was unable to demonstrate a definite difference in drought survival.

The leaf count data recorded from both populations shows that they were significantly affected by drought as measured by proportional leaf loss. This was expected as plants are known to respond to drought stress by leaf senescence i.e. older leaf shedding in response to water deficit (Guo & Guan 2014). The higher rate of survival and lower leaf loss of the Banks Peninsula seedlings, in comparison to the Back Valley seedlings, might reflect greater drought stress tolerance, but again this was non-significant perhaps due to small number of Back Valley seedlings.

### Phytometer experiment

Banks Peninsula is generally considered to have moderately fertile soils, as it is a volcanic region and the parent materials are loess and volcanic rocks (Webb 2008). I hypothesised that *P. obcordatum* is growing in an atypical habitat because the soil composition of the habitat is similar to those of an alluvial flat. This is supported by the fact that other lowland trees which prefer fertile soils grow there (e.g. *Podocarpus totara* and *Prumnopitys taxifolia*) (Wilson 2013; Hutchison 2014).

The phytometer experiment did not support that hypothesis, the Banks Peninsula soil produced the least seedling biomass compared to the alluvial flat habitats and this was significantly different. The Back Valley soil was the most productive of all the soils with a mean biomass production of 4.82 g, as the Owen Valley soil was close with a mean biomass of 3.66 g. The soil from sites which were affected by farming produced the least biomass. When comparing the soils, the two worst performing -Banks Peninsula (0.85 g) and Kaitaia (2.16 g) - came from locations that were part of smaller forest fragments. The Back Valley and Owen Valley sites had better performing plants, even though Owen Valley was also a forest fragment on a farm. The Back Valley habitat is one of the only intact lowland forest habitats in New Zealand (Clarkson & Clarkson 1994; Rance 2007).

Phytometer experiments are a simple and inexpensive method to produce a comparative ranking of different soil fertilities. This phytometer experiment was conducted to assess whether the location of the hillslope population could be attributed to an outstanding general fertility of the hillslope soil. The results of the experiment conclusively demonstrate that this is not the case. It is still possible that there are other soil fertility factors which influence the growth of *P. obcordatum*, which were not demonstrated in this experiment. Radishes were used in this experiment as they are a quick growing species which have experimental precedence (Axmanová et al. 2011); however, the nutrient demands of radishes are unlikely to be identical to *P. obcordatum*.

*Pittosporum obcordatum* appears to be a basicole, only occurring on base-rich substrates (P. de Lange pers.comm). However, the species is not necessarily restricted to the basic habitat it has thus far been described in. *Australopyrum calcis* (Poaceae) is one species that was thought to be restricted to certain soil conditions (calcicole habitats), however, other evidence now suggests they were once occurring on fertile colluvium and alluvium (Molloy 1994). Assumptions about environmental specialisation do not always prove to be correct as they are usually based on field observations, especially when accurate data about historical distributions is lacking. This can mean the ability to tolerate a wider range of habitats in rare species may not be fully understood (Monks & Burrows 2014). For example, soil pH has been an important factor for habitat preference for *Carmichaelia carmichaeliae* (acidic) and *C. glabrescens* (alkaline) (Fabaceae). This strong edaphic preference was presumed to contribute to their scarcity (*C. carmichaeliae* was listed as rare) but records actually show them to have been previously far more widespread (Heenan 1996). Without intensive historical records we can only assume what a species' distribution previously was and that might not be reflected in what it is today (Heenan 1996; Monks & Burrows 2014). As more information is gained and new populations are found we sometimes find that a species is more widespread than previously thought. This was the case with *P. obcordatum*, as most populations have been found in the past 20-30 years and is not as rare as was once thought. Before 1981, it was only known to occur at three locations (Chapter 1). The fact that this population on Banks Peninsula is occurring on a hillslope has widened the description of what is known about its current habitat.

## **Future research and conclusions**

Our understanding of the environmental mechanisms affecting species distributions is incomplete, especially for species in extreme and fluctuating environments (Rogers 1996; Monks & Burrows 2014). It may be that *P. obcordatum* as a species is generally drought tolerant but that the hillslope population from Banks Peninsula is not necessarily more drought tolerant. If so, previous assumptions about the habitat specialisation of *P. obcordatum* must be reconsidered to include the possibility that *P. obcordatum* can occur on hillslopes in other locations. *Teucrium parvifolium* (Lamiaceae), a species occurring in both hillslope and alluvial habitats, is known to be a basicole that grows in similar substrates and habitats as *P. obcordatum* (Boot 1998). It is possible that *P. obcordatum* might be found on hillslopes in other locations.

Alternatively, *P. obcordatum* might be an opportunist, only occurring on a hillslope because the land has been cleared. Furthermore, it could be that *P. obcordatum* requires disturbance and

sufficient light in order to colonise new areas. A single tree is known to be growing on a hillslope in the Catlins, in secondary forest on a farm, which means it might be found on hillslopes in other areas (Rogers et al. 1998). Another possibility again, may be that *P. obcordatum* can access a greater range of habitats if it is provided with enough of another favourable condition. It is known that plants in the northern hemisphere are more likely to survive on north facing slopes (away from the equator), presumably due to increased soil water retention with less exposure (Bennie et al. 2006). This could explain the Banks Peninsula hillslope populations, as the plants are growing on a south facing slope (Bennie et al 2008; Hutchison 2014). Another possibility is that the hillslope location at Banks Peninsula is an extreme habitat, inhospitable to broadleaf species, and similar slopes near other *P. obcordatum* populations are hospitable to broadleaf species which outcompete *P. obcordatum* seedlings (Clarkson & Clarkson 1994).

The hillslope population of *P. obcordatum* on Banks Peninsula is unusual considering what was previously known about its habitat. My results showed no statistical evidence for *P. obcordatum* occurring on a hillslope because the plants had adapted to drier conditions. However, the possibility still exists that Banks Peninsula *P. obcordatum* seedlings are more drought-tolerant than those of other populations as there was a suggestive but non-significant difference in the means with small sample sizes used here. Investigating the hypothesis using larger sample sizes and seedlings from more populations could conclusively answer the question.

The results of the phytometer experiment show that soils occurring on Banks Peninsula were not unusually fertile compared to soils from alluvial flats sites. Overall, the Banks Peninsula soil produced plant biomass that was substantially lower than plant biomass from the three alluvial flat sites. It is unlikely that *P. obcordatum* is occurring on a hillslope because of comparatively high soil fertility at that location.

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## Chapter 4: Synthesis

The primary goal of this thesis was to better understand genetic and ecological factors that could be relevant to the conservation of *Pittosporum obcordatum*. My study is the first conservation genetic study of *P. obcordatum* and adds to a growing number of conservation genetic studies of rare New Zealand plant species (e.g Barnaud & Houliston 2010; Shepherd & Perrie 2011). There have been previous ecological studies on *P. obcordatum* (King 1986; Clarkson 1991; Clarkson & Clarkson 1994), looking into why it was uncommon and describing its ecology. However, this the first study to include two new South Island populations (Banks Peninsula 2012, Owen Valley 2009), and also look into why *P. obcordatum* is growing in an atypical habitat (Banks Peninsula). As such, this study contributes to understanding of the species' habitat specificity.

### New populations

Populations and individuals of *P. obcordatum* are still being discovered. Recent discoveries include Owen Valley (2009), Banks Peninsula (2012) and 120 individuals in Whangarei (2014 private land on the floodplains). There is currently no evidence as to the historical distribution of *P. obcordatum*, so questions remain as to whether the species is naturally uncommon due to habitat specificity, although still affected by habitat loss; or whether it was previously widespread among lowland forests around New Zealand. The genetic structure of *P. obcordatum* found in Chapter 2 suggests there is little or no gene flow between populations currently, but this still does not tell us whether they have strong genetic structure because they are naturally disjunct. This question cannot be easily answered, but any new populations discovered will provide further information that could conceivably produce a clearer picture of the nature of the species' rarity.

The Banks Peninsula population is occurring on a hillside, and one individual has been found on a hillslope in Glenomaru in the Catlins. Additionally, the data from Chapter 3 highlights that *P. obcordatum* might not be constrained to high fertility soils. This means that the habitat of *P. obcordatum* might not be as specialised as previously thought, suggesting that *P. obcordatum* could be found occurring on both hillslopes and less fertile habitats elsewhere. Searching hillslopes adjacent to alluvial flats where the species now occurs would be a useful starting point. The discovery of the Owen Valley population in the Tasman region is a significant find because previously populations were mainly found in eastern lowlands. For this reason, Tasman/Marlborough regions in particular should be targeted for further searches. Compiling

habitat data to undertake species distribution modelling would help highlight other areas where *P. obcordatum* is likely to be found.

Finding more populations may connect the remaining dots as to what the natural range of *P. obcordatum* may be, which would have an impact on conservation decisions. We may be able to identify other spatial patterns in the genetic data, be it more widespread or specialised to specific habitats. The species is presumed now to be unnaturally rare, and further information which did not dispute this would assist in discerning which areas should be targeted for restoration plantings. In the unlikely event that the species was proven to be naturally rare and had enough diversity, conservation would not be such a high priority, or may be approached differently.

### Restoration of small populations

Most known populations of *P. obcordatum* are currently at risk because of small population size. In some cases, this is combined with unequal sex ratios and competition from invasive plants affecting regeneration (Kaitia, A. Townsend pers. comm.) However, as indicated in Chapter 2, small populations contained less genetic diversity than larger populations. Therefore, to mitigate the effects of genetic drift and inbreeding it is recommended that populations of *P. obcordatum* should not decrease in census size any further. To prevent inbreeding depression in outcrossing species such as *P. obcordatum*, populations should have at least 50-100 individuals. For long-term persistence in the wild, 500+ individuals are required (Frankham et al. 2014). The implications raised by the discovery and genetic study of new populations may take us in directions other than focusing on growing the populations we have. However, the data currently at hand supports a recommendation to increase population size by restoration plantings, which would be a form of genetic rescue.

Genetic rescue focuses on restoring genetic diversity and increasing fitness in small populations by augmenting gene flow. For plant species, genetic rescue can take the form of seeds or seedlings planted in a targeted population. Outbreeding depression is a risk for locally adapted populations, and this risk increases with genetic, geographic and environmental distance from other populations (Whiteley et al. 2015). For restoration of genetically unique populations, such as Banks Peninsula and Owen Valley, it is best to source seed from within the population, and from as many individuals as possible to decrease the likelihood of non-random mating (inbreeding) (Frankham et al. 2009). As such, if a population has low genetic diversity, collecting seeds from populations that are geographically close, similar ecologically, and at similar elevations may be useful (Krauss & He 2006). Based on this information, in Chapter 2 I have

made some suggestions on restoring populations studied, summarised in Table 4.1. For example: the three populations from the eastern Wairarapa (Te Kopi, Ahi Paku and Te Kanuka) could benefit from some gene flow amongst them. They are geographically close to each other and genetically similar enough for outbreeding depression to be unlikely.

It is possible that the genetic uniqueness of the Banks Peninsula and the Owen Valley population reflect could local adaptation to environmental conditions, which could be in response to drought or other environmental differences. For this reason, for any restoration for the Banks Peninsula population and in any future plantings in that area, sourcing seeds from other populations is not recommended. Rather, sourcing seeds and seedlings from within the population is important. Additionally, it would be interesting to explore local adaptation in *P. obcordatum* with analyses such as isolation by environment, and to explore this for other disjunct New Zealand plants, in order to understand the role that environment or ecology plays in causing these populations to diverge or speciate (Sexton et al. 2014).

### Land use

Most of the populations of *P. obcordatum* are now occurring in fragments on private land, with the majority numbering <100 individuals. While, as outlined in Chapter 1, most currently known populations have some level of protection now, historically human land use has focused on the lowlands where these populations have been found. The effects of humans on these lowland areas indirectly affects the genetic diversity of small isolated populations (as addressed in Chapter 2).

If there is any chance of genetic restoration succeeding, we also need to address threats posed by land use. Ongoing threats to persistence in the wild include exotic plants, and grazing and trampling by animals. Such threats are particularly dangerous for small populations, but just because a population, such as Back Valley, is large does not mean it should go unprotected. Large populations can still be affected by habitat loss, though this is perhaps more likely in relation to large-scale development. If the proposed Manapouri dam had gone ahead, the Back Valley population would have likely been destroyed (Morrison 1982). The loss of a large population such as this would have a significant impact on the viability of the species.

There are a number of conservation strategies at the land use level, including incentivising landowners to fence off known populations on private land, and supporting access to nurseries for native seedlings. Habitat can be actively restored with the removal of invasive species, and maintained via legislative restrictions that impose conditions upon or disallow development

altogether. However, as with the planning of restoration programs, the effective evaluation and application of conservation strategies at this level requires comprehensive knowledge and careful consideration of population and species habitat and genetic diversity.

### Conclusion

The number and geographic spread of populations and the potential for new discoveries suggest that *P. obcordatum* as a species is not likely to become extinct in the near future. However, size and lack of diversity at the population level indicate there is a real chance of individual populations going locally extinct. The existing habitat and genetic data supports conservation planning, providing strong justification for site-specific restoration planting designed to maximise population size and genetic diversity. However, these measures must also be supported by conservation strategies addressing threats resulting from land use, which should likewise be informed by habitat and genetic data. This is especially important given that interventions at this level often suffer from competing priorities, so it is crucial that measures put in place are effective and clearly justified. The integration of habitat and genetic data, and of biological and land use interventions; and the application of the former to the latter offers the greatest possible chance of maintaining or increasing the population and genetic diversity of *P. obcordatum*, ensuring its adaptation to changing environmental conditions, and its ultimate survival.

Table 4.1 Restoration recommendations based on genetic data from populations of *P. obcordatum* sampled.

| Site              | Monitor population | Restore habitat and population size | Eco-source seeds within population | Eco-source seeds from similar populations |
|-------------------|--------------------|-------------------------------------|------------------------------------|---|
| 1 Back Valley     | Yes                | No                                  | No                                 | No  |
| 2 The Catlins     | Yes                | Yes                                 | Yes                                | Maybe                                     |
| 3 Banks Peninsula | Yes                | Yes                                 | Yes                                | No  |
| 4 Owen Valley     | Yes                | Yes                                 | Yes                                | No  |
| 5 Te Kopi         | Yes                | Yes                                 | Yes                                | Yes                                       |
| 6 Ahi Paku        | Yes                | Yes                                 | Maybe                              | Yes                                       |
| 7 Te Kanuka       | Yes                | Yes                                 | Maybe                              | Yes                                       |
| 8 Paengaroa       | Yes                | Yes                                 | Yes                                | No  |
| 9 Whangarei       | Yes                | Maybe                               | Maybe                              | No  |
| 10 Kaitia         | Yes                | Yes                                 | Yes                                | Yes                                       |

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## Appendix I

Table of Pair-wise Euclidean Distance (km) between sampled populations.

This was created with the geographic distance function in GenAlEx (Peakall & Smouse 2012).

|                   | 1      | 2      | 3       | 4       | 5      | 6      | 7      | 8      | 9     |
|-------------------|--------|--------|---------|---------|--------|--------|--------|--------|-------|
| 1 Back Valley     |        |        |         |         |        |        |        |        |       |
| 2 The Catlins     | 228.00 |        |         |         |        |        |        |        |       |
| 3 Banks Peninsula | 422.55 | 589.93 |         |         |        |        |        |        |       |
| 4 Owen Valley     | 483.40 | 586.67 | 184.02  |         |        |        |        |        |       |
| 5 Te Kopi         | 343.30 | 265.28 | 765.75  | 809.15  |        |        |        |        |       |
| 6 Ahi Paku        | 380.00 | 294.80 | 802.51  | 845.24  | 36.78  |        |        |        |       |
| 7 Te Kanuka       | 364.44 | 278.19 | 786.78  | 828.42  | 21.74  | 17.10  |        |        |       |
| 8 Paengaroa       | 503.73 | 360.04 | 920.86  | 942.39  | 177.91 | 148.87 | 157.32 |        |       |
| 9 Whangarei       | 901.28 | 690.13 | 1275.92 | 1241.11 | 633.64 | 610.80 | 615.69 | 465.47 |       |
| 10 Kaitaia        | 951.96 | 732.77 | 1309.20 | 1309.20 | 706.69 | 686.84 | 690.07 | 545.80 | 99.30 |



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